Recombinant proteins as vaccines and diagnostic antigens for the control of Jembrana disease virus infection in Indonesia

Joshua Lewis

This thesis is presented in fulfilment of the requirements for the degree of Doctor of Philosophy.

2008

Declaration

I declare that this thesis is my own account of my research and contains as its main content, work that has not been submitted for publication or degree at any other educational institution.

Joshua Lewis

Abstract

Jembrana disease virus (JDV) is a lentivirus associated with an acute disease syndrome of Bali cattle (*Bos javanicus*) in Indonesia. Control of Jembrana disease currently involves serological monitoring of infection in endemic areas, restriction of movement of cattle from these areas and ring-vaccination around outbreaks of the disease with a tissue-derived inactivated-virus vaccine.

Earlier investigations at Murdoch University resulted in a vaccine combining a mix of recombinant capsid (CA) and Tat proteins that ameliorated the clinical signs of JDV infection in vaccinated cattle. This thesis reports the development of an alternative novel fused CA and Tat polyprotein antigen emulsified in incomplete Freund's adjuvant (IFA) as a vaccine. This polyprotein could be produced in a single operation at a lower cost than the mix of the 2 individually produced recombinant proteins. The effect of vaccination with this CA and Tat polyprotein vaccine was compared to vaccination with a mixture of individual CA and Tat proteins in small groups of cattle that were subsequently challenged with virulent JDV. It was found that the fused polyprotein vaccine elicited a greater antibody response against both the CA and Tat antigens than a vaccine containing the mix of individual recombinant proteins. In cattle vaccinated with the fused recombinant polyprotein the viral load and lymphocyte response to infection was similar to that in cattle vaccinated with the mix of individual proteins. When the CA and Tat polyprotein vaccine was administered to cattle under field conditions, the vaccine induced minimal side effects and an antibody response to both CA and Tat proteins that persisted for 12 months.

A recombinant protein antigen was produced for ELISA and Western immunoblotting assays that enabled serological detection. This antigen provided an alternative to the whole virus antigen that is currently used in Indonesia for these serological assays. The whole virus antigen is prepared from plasma of JDV-infected cattle using a differential centrifugation technique and is difficult to produce in Indonesia. It was found that a recombinant full length CA (p26) protein antigen used in an ELISA provided sensitivity and specificity equivalent to that obtained by Western immunoblotting with the whole virus antigen. This recombinant CA protein antigen is now used in routine serological assays for detection of JDV in Indonesia.

Acknowledgements

There are a number of people who have been instrumental in helping, guiding and coaxing me through the course of my PhD whilst maintaining my sanity and good humour.

Firstly I must thank Moira Desport for her constant help, supervision and friendship throughout the project. Throughout the course of the PhD Moira was always discussing results and the future direction of my research over a coffee and cake and for this I will be eternally grateful. I must also thank Graham Wilcox who has always been on hand for guidance and understanding.

I would also like to thank the academics at Murdoch University for putting up with my constant questions in particular Cassandra James, Sandy McLachlan and Phil Clarke. There have also been a number of past and present PhD students who have helped me immensely so thank you Meredith Stewart (my mentor during honours and all round lab guru), Michael Bananzis, Lucy Woolford, Mark Bennett, Judhi Rachmat, Linda McInness, Susannah Cleary and Ryan Jefferies.

A number of people in the Veterinary Virology group have helped me along either with a piece of timely advice or a piece of cake to keep my spirits up. So thanks must go to Linda Davies (for drinking my bad coffee), Tegan McNab (for fattening me up with home baked cakes), Masa Tenaya (for keeping me company on weekends at the office), Will Ditcham (for lots and lots of advice and help), Nicolai Bonne (for reminding me to chill out), Mark O'Dea (Ag department guru) and Andrew 'Bacon Sarnie' Hughes.

Terima Kasih Banyak to everyone at the Disease Investigation Centre in Denpasar in particular, Masa Tenaya, Nining Hartaningsih, Putu Agustini, Widia and Mundra for all their excellent work in running the animal trials in Indonesia.

To my family in particular my parents Ian and Ani for your unending support and love I am truly grateful and blessed. To my brothers Matt, Nic and Simon thanks for keeping me grounded and to my nieces and nephews for keeping me smiling.

Finally I must thank my beautiful fiancé Jesslyn for always supporting and loving me throughout the whole process even when I was stressed or grouchy. I could never have finished this without your support and smiles *Wo ai ni*.

Index

Abbreviations used in thesis

Ab - antibody

APC - antigen presenting cell

bp - base pair

BIV - *Bovine immunodeficiency virus*

BLV - *Bovine leukaemia virus*

BSA - bovine serum albumin

BSV - *Bovine syncytial virus*

CA - capsid protein

CAEV - *Caprine arthritis-encephalitis virus*

CNS - central nervous system

CTD - C-terminal domain

dsDNA - double-stranded DNA

EIAV - *Equine infectious anaemia virus*

ELISA - enzyme linked immunosorbent assay

Env - envelope glycoprotein precursor

EU -endotoxin unit

FBL - foetal bovine lung cells

FIV - *Feline immunodeficiency virus*

GST - glutathione-S-transferase

HIV-1 - *Human immunodeficiency virus* type 1

HIV-2 - *Human immunodeficiency virus* type 2

IFA - incomplete Freund's adjuvant

IN - integrase

IPTG - isopropyl-ß-D-thiogalactopyranoside

ISH	- *in-situ* hybridization
IU	-international endotoxin unit
JDV	- Jembrana disease virus
LTR	- long terminal repeat
LPS	- lipopolysaccharide
MA	- matrix protein
MAb	- monoclonal antibody
MHR	- major homology region
MOA	- mineral oil adjuvant
NC	- nucleocapsid protein
NTD	- N-terminal domain
OD	- optical density
OMVV	- ovine maedi visna virus
ORF	- open reading frame
PBMC	- peripheral blood mononuclear cells
PBS	- phosphate buffered saline
PBS/T	- phosphate buffered saline/Tween 20
PCR	- polymerase chain reaction
PMSF	-phenyl methyl sulfonyl fluoride
PR	- protease
qPCR	- real-time (quantitative) PCR
RBC	- red blood cells
Rev	- regulator of virus expression protein
RT	- reverse transcriptase
SDS-PAGE	-sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIV	- *Simian immunodeficiency virus*

S/HIV	- Simian/Human immunodeficiency virus
ssRNA	- single-stranded RNA
SU	- surface envelope protein
TAR	- trans-activation response region
Tat	- transactivator of transcription protein
TEMED	- N, N, N', N'-tetra methyl ethylene diamine
TM	- transmembrane protein
TRAIL	- tumour necrosis factor-related apoptosis-induced ligand
Vif	- viral infectivity factor protein
WBC	- white blood cells

List of units

°C	-degrees Celsius
µg	-micrograms
µl	-microlitre
µm	-micrometre
µM	-micromolar
ρmol	-picomoles
bp	-base pairs
d	-days
EU	-endotoxin units
g	-grams
g	-times gravity
h	-hour(s)
ID_{50}	-50% infectious dose
kb	-kilobases
kDa	-kiloDalton
kV	-kilovolts
M	-molar
mg	-milligrams
min	-minute(s)
mL	-millilitre
mM	-millimolar
ng	-nanograms
nm	-nanometre
OD	-optical density
rpm	-revolutions per minute
U	-units of enzyme activity
V	-volts
v/v	-volume per volume
w/v	-weight per volume

Chapter 1. Introduction

Jembrana disease in Bali cattle (*Bos javanicus*) was first detected on the island of Bali in Indonesia in 1964. The initial outbreak occurred in the Jembrana district and soon spread to surrounding districts (Adiwinata, 1968; Budiarso and Hardjosworo, 1976). Within a year the disease had spread throughout Bali and had killed an estimated 26,000 cattle out of the 300,000 Bali cattle on the Island. The disease has since spread to and is now endemic in the Bali cattle population of the islands of Java, Sumatra and the Indonesian provinces of Kalimantan in Borneo island (Hartaningsih et al., 1993; Soeharsono, 1995; Soeharsono and Temadja, 1995). The aetiological agent was identified as a virus and designated *Jembrana disease virus* (JDV) and then subsequently identified as a lentivirus genetically and antigenically similar to *Bovine immunodeficiency virus* (BIV) in 1993 (Chadwick et al., 1995a; Kertayadnya, 1995; Kertayadnya et al., 1993; Wilcox et al., 1995). The genetic characterization of the virus has provided the opportunity for the utilization of molecular biological techniques for the development of improved diagnostic and control procedures.

The control of Jembrana disease in Indonesia has been based primarily on restrictions on the movement of cattle out of endemic regions to disease-free areas. This has been supplemented by the development of a whole virus immunoassay to monitor the distribution of the disease in Indonesia and subsequently by the use of a tissue-derived inactivated virus vaccine prepared from the spleen of experimentally infected animals (Hartaningsih et al., 1995b). This vaccine has been shown to ameliorate the disease and prevent mortalities in vaccinated cattle but due to production issues and cost is only produced in small amounts and used only to ring-vaccinate around outbreaks to slow the spread of the disease to adjacent areas (Hartaningsih et al., 2001). A second generation vaccine using the bacterially expressed JDV Capsid and Transactivator of Transcription glutathione-S-transferase (GST) tagged proteins was also reported to ameliorate Jembrana disease and prevent mortalities in experimentally infected cattle (Ditcham, 2007).

The major objectives of the studies reported in this thesis were the development and use of recombinant proteins expressed with a bacterial expression system, to replace the whole virus immunoassays currently used in Indonesia and the development and

testing of an alternate recombinant protein vaccine that could be produced from a single construct.

To provide a background for the research reported in this thesis, a review of the literature relating to lentiviruses and Jembrana disease was undertaken and is presented in Chapter 2. This review includes background information on lentiviruses, other animal lentiviruses with emphasis on their diagnosis and control by vaccination, historical aspects of Jembrana disease and BIV, and on potential systems for recombinant protein production and their use for the production of diagnostic reagents and vaccines.

In Chapter 3, the development of recombinant proteins expressed in *Escherichia coli* and used as antigens to replace the current whole virus antigen used in immunoassays is described. This Chapter also includes a description of the Western immunoblot assay and ELISA utilising the recombinant protein as antigen for the detection of CA and Tat antibody in cattle, and a comparison of the sensitivity and specificity of the assays utilizing these recombinant proteins to those utilizing the whole virus antigen.

To improve on previous methods used to produce individual recombinant CA and Tat proteins of JDV, that were combined and used as a vaccine for Jembrana disease (Ditcham, 2007), a system for the expression in *E. coli* of both these proteins as a GST-tagged polyprotein (CA/Tat-GST) from a single construct was developed and is reported in Chapter 4. This chapter also includes a description of methods used for the optimization of induction and purification of the protein, *in vitro* cytotoxicity of the recombinant products, and methods used for determining purity and contamination of the polyprotein with nucleic acid and endotoxins.

Chapter 5 reports the use of the CA/Tat-GST polyprotein as a vaccine for inhibition of the effects of JDV infection in small groups of cattle. The efficacy of the polyprotein as a vaccine was compared to the use of a vaccine containing a mixture of individually prepared CA-GST and Tat-GST proteins.

Chapter 6 describes the use of the CA/Tat-GST polyprotein in a large scale trial involving 100 vaccinated cattle and 100 control cattle over a 12 month period. This included an examination of possible adverse clinical effects of vaccination and the subsequent antibody response to vaccination.

A general discussion of the positive outcomes of the research reported in the thesis, of the limitations of the research and potential methods for improvement of the outcomes is presented in Chapter 7.

Chapter 2. Review of literature

This chapter reviews the current literature relevant to the objectives of the research undertaken and reported in this thesis on Jembrana disease and Jembrana disease virus (JDV). JDV is a member of the bovine lentiviruses within the family *Retroviridae*, so an overview of the characteristics of retroviruses, and lentiviruses in particular is given to provide background information on the structure, genome, antigenicity and replication of this group of viruses. An understanding of the comparative pathogenesis, viral structure, genomics and antibody response to the lentiviruses was considered crucial to an understanding of the significance of the investigations reported in this thesis.

The nomenclature used in this thesis for viral genes and proteins is that suggested by (Fauquet and Mayo, 2001; Pringle, 1999) where gene names are in lower case and italics e.g. *env.* The abbreviations for the encoded proteins have at least the initial letter in uppercase, e.g. Tat, Env and TM, and are not italicized.

Retroviruses

The family *Retroviridae* is comprised of a diverse group of viruses divided into 7 genera: *Alpharetrovirus, Betaretrovirus, Deltaretrovirus, Gammaretrovirus, Epsilonretrovirus, Spumavirus* and *Lentivirus*. These are all animal viruses that use RNA as their genetic material within the virion and DNA as the genetic information within the infected host cell. Due to their dual genetic information systems, retroviruses have acquired characteristics of both DNA and RNA viruses (Flint et al., 2000). An important feature of retroviruses is therefore their ability to use the enzyme reverse transcriptase to produce a proviral DNA (proviral) copy of the RNA genome, which is subsequently integrated into the host genome. Integration of the virus genome into the cellular genome makes retroviruses highly efficient molecular parasites. They can be subdivided into oncogenic or non-oncogenic types based on their ability to cause any form of neoplasia in the infected host species, and all but the *Spumavirus* and *Lentivirus* genera have oncogenic potential (Levy, 1993a). Large-scale research into animal and human retroviruses has intensified in the last 2 decades, prompted by the discovery of *Human immunodeficiency virus* (HIV).

Lentiviruses

Lentiviruses are exogenous viruses that are biologically and genetically distinct from other retroviruses (Pantaleo and Walker, 2000). There are at least 8 known species in humans and animals, with HIV being the best (Foley, 1998). Other known species are *Maedi visna virus* (MVV) in sheep, *Caprine arthritis encephalitis virus* (CAEV) in goats, *Simian immunodeficiency virus* (SIV) in non-human primates, *Feline immunodeficiency virus* (FIV) in wild and domestic cats, *Equine infectious anaemia* virus (EIAV) in various species of *Equidae*, and the related bovine species *Bovine immunodeficiency virus* (BIV) and JDV.

Lentiviruses typically produce an initial mild acute disease syndrome soon after infection, followed by apparent recovery and a prolonged asymptomatic phase, followed by a progressive chronic symptomatic phase often leading to death. The exceptions to this include BIV, that seems to be associated with sub-clinical infection only (Gonda, 1992), and JDV and the $SIV_{smmPBj14}$ that cause severe and sometimes fatal acute disease syndromes after short incubation periods (Campbell, 1995; Soeharsono et al., 1995a; Wilcox et al., 1995). Lentiviruses principally infect cells of the immune system including macrophages, B-cells and T-cells, which leads to a compromised immune system and subsequent secondary infections.

The lentivirus genome encodes 3 large polyproteins from the 3 major open reading frames (ORF): *gag* (derived from group antigen) encoding structural proteins), *pol* (derived from polymerase) encoding viral enzymes, and *env* (derived from envelope) encoding envelope glycoprotein involved with virus-host cell interactions as shown in Figures 2.1 and 2.2. Lentiviruses also encode a number of accessory proteins from genes located between the *pol* ORF and the 3' LTR that have important regulatory functions during viral replication; these genes are characteristic of and unique to lentiviruses, and some of these are unique to the individual lentiviruses (Flint et al., 2000; Levy, 1993a). The common lentivirus accessory genes are *vif* encoding Vif (viral infectivity factor), *tat* encoding Tat (trans-activator of transcription), *rev* encoding Rev (regulator of expression of virus) and *nef* (negative factor) which is unique to primate lentiviruses (Miller et al., 2000b).

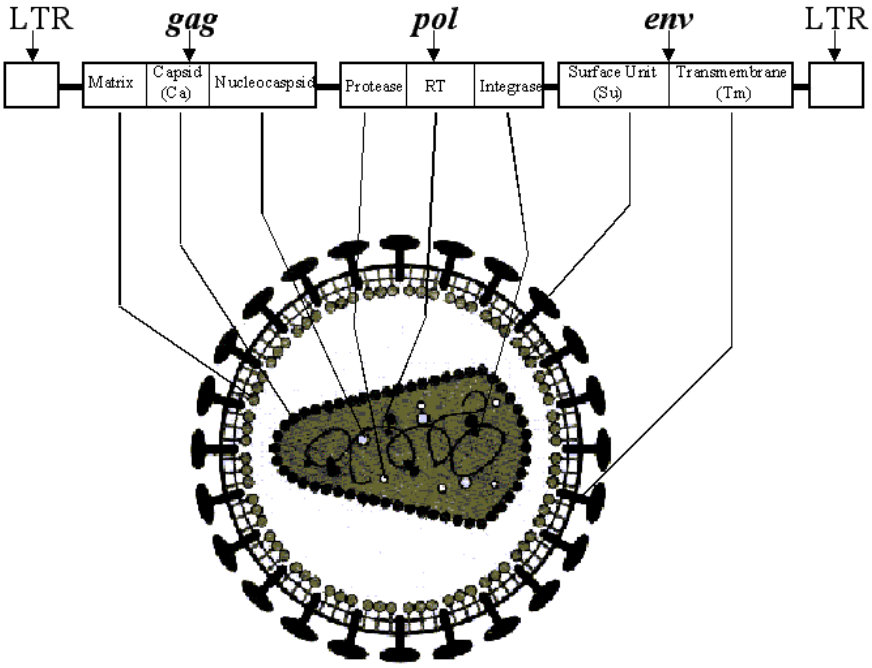


Figure 2.1. Schematic illustration of the morphology and genome structure of lentiviruses showing the 3 main open reading frames (ORF). The genes encoding the accessory proteins are not shown. The diagram is an adaptation of that used by Gonda (1992) to describe the structure of BIV.

Pathogenesis of lentivirus diseases

Knowledge of the replication of lentiviruses is crucial for an understanding of the progression of the diseases they induce and the host response to infection. The first step in the infection process is the entry of the virion into the host cell, facilitated by high affinity binding of the surface envelope (SU) glycoprotein to specific viral cell receptors on the surface of the susceptible host cell (Gonda et al., 1994; Pantaleo and Walker, 2000). Once attached to the host cell, viral entry proceeds by fusion of the viral envelope with the membrane of the cell, enabling the virus core comprising of NC and CA (residual structure formed by loss of the matrix protein and envelope glycoprotein) to enter the cytoplasm of the host cell, wherein the single-stranded viral RNA is reverse transcribed into a double-stranded viral DNA utilising the intravirion virus-encoded reverse transcriptase. This double-stranded DNA is transported to the nucleus and integrated into the host cell's chromosomes via an intravirion virus-encoded integrase (Flint et al., 2000). The integrated virus double-stranded DNA (provirus DNA) is duplicated each time the cell divides, thus maintaining the virus

genome through generations of cell division. Upon activation of key cellular and exogenous signals (yet to be elucidated) viral expression can be initiated (Gonda, 1992). Once the host cellular enzymes transcribe the viral DNA into mRNA, using splicing mechanisms, the RNA transcripts are transported to the cytoplasm where they are translated to non-glycosylated proteins by free ribosomes or to glycosylated proteins by endoplasmic reticulum bound ribosomes and the Golgi apparatus. The virus components are then assembled in the cytoplasm and are released from the cell by a budding process when the virus acquires the envelope and further maturation can occur after release of the virus from the cell (Hovden, 2001) (Figure 2.2).

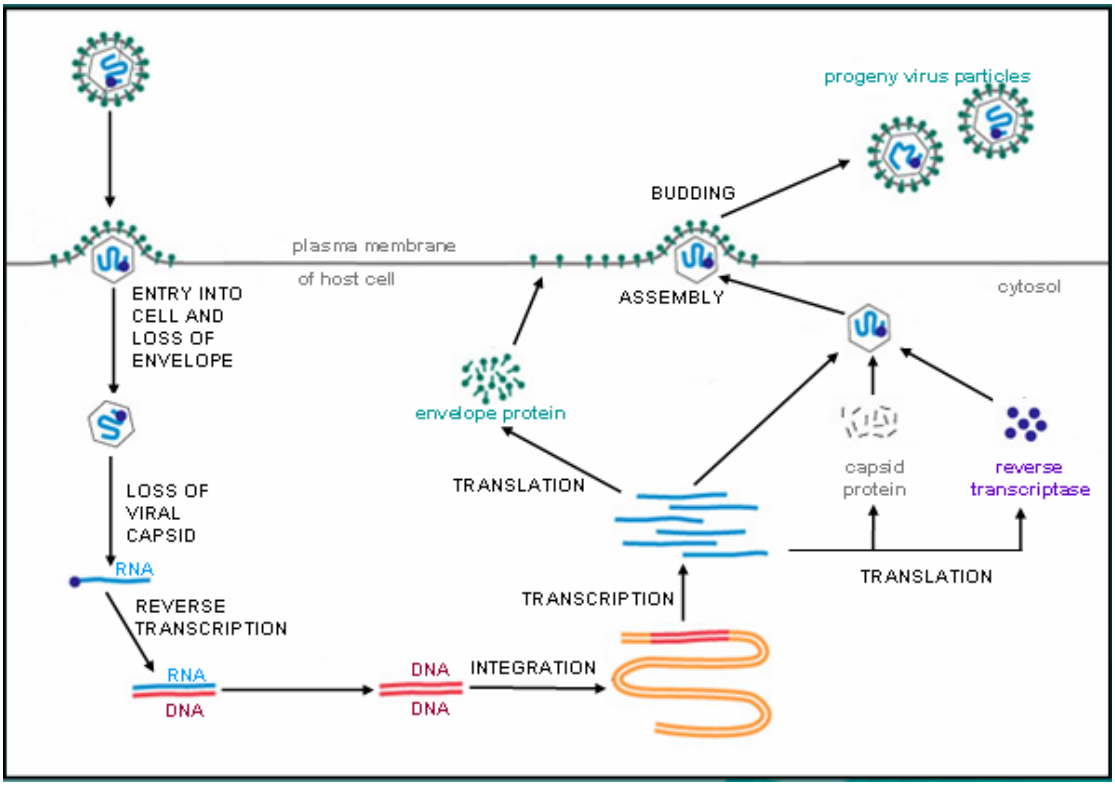


Figure 2.2. Schematic diagram illustrating the series of events that occur during lentivirus replication cycle within the host cell. Once the virus binds and enters the host cell leading to the loss of the envelope and capsid surrounding the ssRNA. The ssRNA is then reverse transcribed into dsDNA and integrated into the host chromosome. Once cell division occurs, viral mRNA is transcribed and then translation of the viral products follows. Unlike AIDS inducing lentiviruses which do not form intracytoplasmic particles before JDV viral components are then assembled and bud from the host cell to infect other susceptible cells (adapted from Stewart 2005).

Immune response to lentiviral infections

An immune response to viruses occurs when the physical and chemical barriers such as skin, mucous secretion, acidity and tears are overcome or bypassed by the virus (Cotran et al., 1999). Once these defences are breached, there are 3 main processes involved in the immune response: recognition of the virus, activation of the immune response, and control of the immune response once the viral infection is cleared. The immune response that occurs can be divided into 2 main types: one associated with the innate immune system and one associated with the adaptive immune system (Flint et al., 2000).

The innate immune system is a non-specific system that is being continuously activated in response to infection by viruses (Cotran et al., 1999). Once triggered, the innate immune system controls interferon production, complement and inflammatory responses. Interferons can induce an anti-viral state in neighbouring cells that will inhibit virus replication, and play a role in the clearance of infected cells by activating the complement cascade, natural killer cells (NK), macrophages or neutrophils (Narayan et al., 1982). The inflammatory response is usually only strongly activated by cytopathic viruses that cause significant cell damage. The innate immune response is vital as it is the body's first response to the virus, can be activated within hours of infection and is often a precursor to the adaptive immune response (Cotran et al., 1999).

In contrast to the innate immune system, the adaptive immune response is specific and tends to be much slower, taking days to weeks to reach peak activity (Roitt and Delves, 2002). The increased response time is the requirement for activation of antigen-presenting cells (APC) before the process of differentiation and maturation of the response can occur. The adaptive immune response is categorized into 2 main classes: humoral and cell-mediated.

The humoral response is the presentation of antigens to immature B-lymphocytes that then differentiate into plasma/antibody-secreting cells. The 5 classes of antibodies produced are IgA, IgM, IgD, IgE and IgG, all of which have different antigen binding properties and biological roles (Roitt and Delves, 2002). The classes that appear to be important in viral infections are IgA (involved in mucosal viral defence), IgM (important in early viral agglutination and activation of the complement cascade) and

IgG (the prime effector antibody class in viral clearance) (Cavacini et al., 2003). IgG is the major class of antibody produced in response to infection: 75% of all antibodies present in plasma are of this subtype. IgG has a half-life of 1-3 weeks (depending on the subclass) and is produced predominantly after a second exposure to an antigen. There are 4 subclasses of IgG (IgG1, IgG2, IgG3 and IgG4), with IgG1 being the most abundant subclass (Flint et al., 2000). In HIV-1 infections, IgG1 and IgG3 are involved in binding and neutralization of virus (Cavacini et al., 2003). Some classes and subclasses of antibody are important regulators of cell-mediated immunity and inflammation (Casadevall and Pirofski, 2003) whilst others such as IgM, IgG and IgA can activate the complement cascade leading to clearance of virally infected cells (Casadevall and Pirofski, 2004).

The cell-mediated immune response is brought about by the actions of the helper or cytotoxic T lymphocytes. These cytotoxic T lymphocytes are activated by APC that either destroy infected host cells directly or do so by antibody-dependant cell-mediated cytotoxicity. The helper T lymphocytes release factors which activate and enhance the activity of other T and B lymphocytes and also limit cell division in infected tissues, thus preventing viral spread (Roitt and Delves, 2002).

Small ruminant lentiviruses

Although the primate lentiviruses are the best characterized of all the lentiviruses, they were not the first lentiviruses identified. The small ruminant lentivirus OMVV was discovered first and is described below.

Ovine maedi-visna virus (OMVV)

OMVV infects sheep causing the 2 diseases “maedi” and “visna,” affecting the central nervous system (CNS) and respiratory system, respectively (Dawson, 1987). These diseases appear to be confined to sheep in the northern hemisphere (Campbell, 1995) and have not been identified in Australia.

OMVV is a spherical enveloped virion 80-100 nm in diameter which is morphologically similar to other lentiviruses (Levy, 1993b) The virus replicates primarily in circulating monocytes and tissue macrophages. After infection of sheep, typically by transfer of infected blood, or in lambs by ingestion of virus-infected milk from infected dams during suckling, viraemia can be detected 2-6 weeks post-

infection and continues until death of the animal. Clinical signs do not develop for a prolonged period (months to years) post-infection. Maedi is characterized by chronic respiratory disease and mastitis in older ewes, and visna by meningoencephalitis and arthritis (Dawson, 1987).

Within 6-10 days of infection, there is a detectable antibody response to the CA and predicted MA (p16) proteins of OMVV. Not all infected animals develop a humoral immune response to the Env proteins, and sheep with chronic lesions may not have a detectable antibody response to virus proteins including p14, the predicted MA, and in some cases even CA. This total lack of antibody response to virus antigens in chronically infected animals makes diagnosis difficult (Houwers and Nauta, 1989).

Currently, there are no commercial vaccines available for OMVV.

Caprine arthritis-encephalitis virus (CAEV)

CAEV is genetically and antigenically related to OMVV, producing disease syndromes in goats that are similar to those induced by OMVV in sheep (Foley, 1998). CAEV infection is most common in high-density dairy goat production areas in America and Europe but it has also been diagnosed in Africa, Australia and New Zealand (Levy, 1993a). The infectious virus is an enveloped spherical virion 110 nm in diameter (Levy, 1993b).

Transmission of the CAEV between animals occurs via milk, blood, saliva or urogenital secretions (Logan et al., 2004). Upon entry into a susceptible animal by ingestion, the virus is absorbed into the small intestine wall and then infects mononuclear cells in the region; when these mononuclear cells mature into macrophages, the virus is activated and replication occurs (Narayan et al., 1982). The clinical manifestations of infection are similar to those of OMVV infection in sheep, and principal lesions occur in the CNS resulting in encephalitis (observed mainly in kids) or chronic arthritis (observed mainly in adults). Other manifestations of infection can also occur, including pneumonia, mastitis, secondary infections and progressive weight loss; death is inevitable once clinical signs develop (Cheevers et al., 1988).

Although virus infection results in a strong humoral and cell-mediated response, typical of the response in other lentivirus infections, this fails to eliminate the virus from the infected animal (Narayan et al., 1982). Serologically, in CAEV infections the

p28 CA is the most immunodominant protein but antibody against the TM and SU proteins are also detectable in infected animals (Bertoni et al., 2000).

Equine infectious anaemia virus (EIAV)

Equine infectious anaemia was the first lentivirus-induced disease reported, described initially in horses in France in 1904. The virus, a circular virion approximately 100 nm in diameter with many envelope projections was, however, only characterized recently (Levy, 1993b). Infections of *Equidae* with EIAV have been reported throughout the world but infection appears more prevalent in warmer climates, possibly because EIAV is a blood-borne virus mechanically transmitted by parasitic vectors such as biting flies (family *Tabanidae*) although it can also be transmitted iatrogenically via surgical equipment or syringes used on multiple animals (Foil et al., 1983).

Like CAEV, the virus replicates in monocytes and macrophages (Oaks et al., 1998) and endothelial cells (Oaks et al., 1999). The anaemia is immunologically-mediated: virions are released from infected cells and bind to red blood cells (RBCs) causing them to be targeted by the immune system, resulting in lysis of the RBCs and subsequent anaemia (Levy, 1993b). Following the initial infection, neutralizing antibody is produced and virus replication declines. This state continues until another variant of the virus is produced that can evade the pre-existing neutralizing antibody, initiating a new cycle of virus replication and disease (Sellon et al., 1996). Each EIAV creates 10-20 copies of proviral DNA which are integrated into the host chromosomes, enabling persistence of the virus in infected animals (Levy, 1993b).

There are 2 recognized clinical phases of equine infectious anaemia, a highly pathogenic acute phase with varying degrees of fever and anaemia, which leads to death in less than 5% of infected animals. Recovery from this initial acute phase is followed by recurrent cycles of viraemia and anaemia, but this is not seen in all infected horses. In this recurrent phase, infected animals have periodic occurrences of disease characterized by clinical signs of fever, decreased fertility, petechial haemorrhages, leucopenia, oedema, loss of weight and condition, and depression. Between each febrile disease period there is a sub-clinical infection where horses have low level circulating virus and antibody (Levy, 1993b).

Serologically, EIAV-infected animals develop a response against the p26 CA (Ball et al., 1992), the gp45 TM and gp90 SU antigens (Chong et al., 1991).

Feline immunodeficiency virus (FIV)

FIV is a spherical 105-125 nm virion with poorly defined envelope projections (Levy, 1993c) first identified in California in 1986 (Pedersen et al., 1987).

The main route of transmission of FIV is the inoculation of saliva from the bite of infected cats, but other routes of infection including *in utero* and perinatal transmission via milk are also possible (Torten et al., 1991). FIV infects not only domestic but also large wild cats in game parks in eastern Africa and in captivity throughout the world, and can cause a disease that is very closely related to that caused by HIV-1 (Brown et al., 1994; Burkhard and Dean, 2003; Langley et al., 1994; Olmsted et al., 1992). The progression of disease associated with FIV infection is said to be an important model for HIV infection studies (Campbell, 1995).

Similar to HIV and SIV, and unlike the macrophage-tropic OMVV, CAEV and EIAV, FIV primarily infects macrophages and lymphocytes. Following infection, animals develops a chronic infection that is generally associated with 3 clinical stages that are very similar to those seen in HIV infections: an initial acute phase associated with fever and lymphadenopathy (enlarged lymph nodes); a subsequent second sub-clinical phase lasting months to years with varying viral plasma load; a third active phase in which there is a progressive destruction of the lymphocytes and subsequent immunodeficiency. Cats progressing to the final chronic stage develop many secondary or opportunistic infections that eventually result in death (Torten et al., 1991).

Serologically, a detectable antibody response against the Gag and Env proteins occurs but some confusion exists as to which antibodies appear first (Burkhard and Dean, 2003; Torten et al., 1991).

Primate lentiviruses

These lentiviruses are the most intensively studied of all the lentiviruses. The simian viruses provide a valuable model for comparative research purposes of HIV infection, and both the simian and human viruses provide a valuable insight into the pathogenic mechanisms involved in non-primate lentivirus infections.

Simian immunodeficiency virus (SIV)

Several retroviruses have been detected in numerous monkey species throughout Africa, some of which have been associated with disease including an AIDS-like syndrome called Simian AIDS (SAIDS) (Levy, 1993c). These lentiviruses are most closely related to the human lentiviruses, and there is strong evidence that the human lentiviruses were derived from SIV infections of non-human primates (Foley, 1998). The first simian retrovirus identified was a D type retrovirus (a deltaretrovirus) (Levy, 1993c). Another retrovirus was identified subsequently, originally designated Simian T lymphotropic virus strain III but later redesignated SIV. Numerous strains of SIV exist, each with differing characteristics and disease pathogenesis, however they are generally classified by the species infected e.g. SIV_{smm} for the SIV detected in sooty mangabeys (Levy, 1993c).

Morphologically, SIV have an irregular roughly circular shape and are approximately 125-165 nm in diameter with outer envelope projections (Levy, 1993c). The method of their transmission in monkey populations in the wild is arguable but there is strong evidence that, like HIV, the main method of transmission is sexual (Milman and Sharma, 1994). The cell types infected are primarilt monocyte/macrophages and T cells bearing the CD4+ receptor similar to HIV (Chakrabarti et al., 1994).

In some circumstances, a disease with a remarkably similar pathogenesis to HIV ensues post-infection with some SIV biotypes, characterized by typical lymphoid depletions, immunodeficiency and fatal secondary infections (Levy, 1993c). Many SIV biotypes in African primates produce sub-clinical infections and the viruses appear adapted to their primary host, with disease occurring only when virus is inoculated into a heterologous host, for example when SIV_{smm} was inoculated into Asian macaques (Levy, 1993c). An acute form of SIV due to a mutant strain of SIV_{smm}, designated $SIV_{smmPBj14}$, was identified that caused an acutely lethal disease within 8-12 days of infection in Asian macaques. Antibodies against the Gag and Env proteins have been detected in infected animals, and neutralising epitopes in the SU protein identified (Cole et al., 1998; Kannagi et al., 1986).

Human immunodeficiency virus (HIV)

HIV is the most characterized lentivirus and there are 2 types, HIV-1 and HIV-2. Both of these subtypes cause AIDS in affected individuals (Andersson et al., 1997; Cotran

et al., 1999). The HIV-1 virus is 80-100 nm in diameter with a spherical shape and is covered with small surface projections on the outer envelope of the virion (Levy, 1993d).

Transmission of HIV involves the transmission of virus in body fluids of infected individuals via infected blood, sexual contact, vertical transmission and sharing of contaminated needles (Cotran et al., 1999). Entry of HIV into the body occurs through mucosal surfaces where susceptible T cells and macrophages (those bearing the CD4+ receptor) in the region are infected. After entry into the cell, virus replicates and newly formed virions are released into the lymph or circulatory system, leading to infection of other susceptible T cells (Flint et al., 2000). Following entry into the lymph and circulatory systems, 3 stages of the disease process ensue. The first stage is an acute infection 4-6 weeks after the initial infection, characterized by large numbers of infectious virus particles produced in the lymphatic system, associated with swollen lymph nodes and flu-like symptoms. Recovery from this phase is followed by a second or asymptomatic phase characterized by a steady decline in CD4+ cells. This phase of infection may persist for as few as 4 months but may be as long as several years. The third (symptomatic) stage of disease is associated with changes in the phenotype of the virus and increased replication of virus, resulting in marked depletion of CD4+ cells and immunosuppression, leading to the clinical condition AIDS (Figure 2.3). Clinical manifestations of the third stage are lymph node enlargement, chronic immune stimulation and the production of auto-antibodies (Cotran et al., 1999). Immunosuppression leads to opportunistic infections by organisms such as *Toxoplasma gondii, Cryptococcus neoformans* and *Pneumocystis carinii.* Other pathologic conditions associated with HIV infection and associated with the immunosuppression include cancers such as Kaposi's sarcoma, B cell lymphomas and anogenital carcinomas, in addition to damage occurring to the gastrointestinal system, visceral organs, and haematopoietic system. Neurological disorders as a consequence of HIV infection of neural cells are also seen (Georgsson, 1994). The neurological lesions, in addition to all the other symptoms as a consequence of immunosuppression, cause a general deterioration of health and eventually result in death if antiviral therapy is not initiated and maintained (Flint et al., 2000). Antibodies against all the HIV viral proteins have been reported although they are rarely seen

except against the CA (p24), SU (gp120), TM (gp41) and Rev (p23) proteins (Cotran et al., 1999).

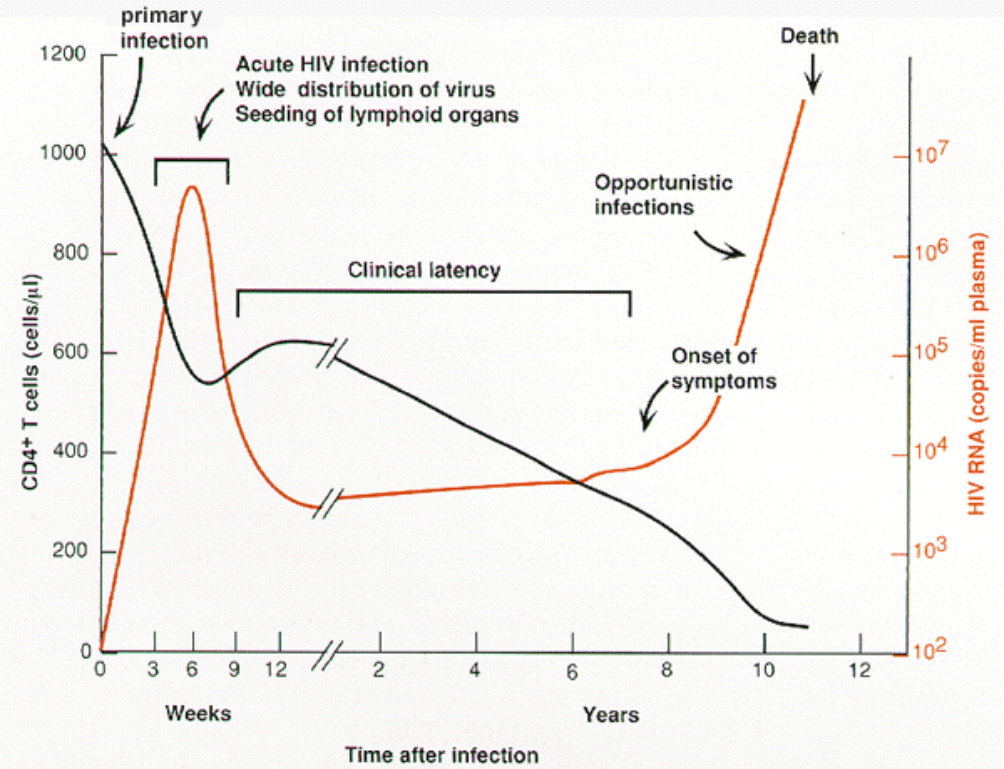


Figure 2.3. Course of a typical HIV-1 infection, the best characterized of all lentivirus infections. After primary infection an acute disease process occurs soon after infection that is associated with a transient high plasma virus load and a decrease in $CD4^+$ cells. This is followed by a prolonged sub-clinical period, with a progressive decrease in total $CD4^+$ cells an eventual increase in virus load leading to the onset of immunosuppression and opportunistic secondary infections (AIDS). Adapted from Weiss (1993).

Bovine lentiviruses

There are currently only 2 known bovine lentiviruses: BIV which produces a mild or sub-clinical disease syndrome, and JDV which produces an acutely pathogenic infection particularly of Bali cattle. The 2 viruses share significant nucleotide and amino acid identity.

Bovine immunodeficiency virus (BIV)

History

BIV was initially identified in a dairy cow in Louisiana (Van Der Maaten et al., 1972). This initial report was virtually ignored by the scientific community until HIV infections became a global issue and the bovine virus was identified as a lentivirus with a genomic organization similar to HIV (Gonda et al., 1987). This original strain of BIV was designated the R29 strain, and other strains have since been detected in America and in many other countries (Scobie et al., 2001; Suarez et al., 1993). Burkala *et al.* (1999) presented evidence that BIV is present in Australia with roughly 3.8% of animals testing seropositive to BIV and JDV recombinant protein antigens.

Transmission and host species

The exact mode of transmission of BIV is yet to be confirmed, but it was suggested that the primary routes of infection occur via virus in blood, milk and other body fluids, and by vertical transmission (Gonda et al., 1994). Other suggested routes of infection include contaminated instruments or via virus-contaminated colostrums (Levy, 1993b). The range of cattle species susceptible to BIV infection is yet to be determined but it appears that most cattle breeds are susceptible (Gonda, 1992). Other species of animals used in transmission studies to test the host range of the BIV R29 strain include New Zealand white rabbits, which develop a persistent viraemia after a single injection of BIV infected cells. Rabbits develop a BIV-specific humoral immune response and virus can be detected in the spleen, lymph and peripheral blood leucocytes, indicating that BIV in rabbits affects cells of the immune system (Pifat et al., 1992). Experimental infections of sheep and goats with the R29 strain have caused these animals to seroconvert within 2 weeks of the inoculation of virus. However, some doubt still exists as to whether these animals actually became infected or produce antibodies to the administered viral components (Jacobs, 1995). These results, indicating susceptibility of not only cattle, but also sheep, goats and rabbits to the same lentivirus are unusual as most lentiviruses produce infection in only a single or closely related host species.

Physical characteristics of virus

BIV is one of 3 syncytial-inducing viruses found in cattle and is 80-100 nm in diameter with short surface envelope projections (Levy, 1993b). The genome is 8,482 bases long (Florida strain 127; BIV 127) and analysis of the few strains that have been detected suggests the virus has been genetically stable over time (Carpenter et al., 2000) unlike most lentiviruses which have a relatively fluid genetic and therefore antigenic structure (Garvey et al., 1990). The primary cell types infected with the virus appears to be B-lymphocytes and macrophages but proviral DNA is also present in CD3+, CD4+, monocytes, T cells, neurons, smooth muscle cells, endothelial cells, microglial cells and CD8+ cells, which indicates that the virus has broad cell tropism (Heaton et al., 1998; Whetstone et al., 1997; Zhang et al., 1997a).

The BIV genome has the obligatory *gag, pol* and *env* genes that are present in all retroviruses but it also encodes additional accessory genes characteristic of lentiviruses and including *vp2* (also called *vpw*), *vp3* (also called *vpy*), *rev, p2L, p3, p2, p7, tmx, tat* and *vif* (Gonda et al., 1994). The Gag precursor polyprotein (55 kDa) is cleaved to produce MA (16 kDa), CA (26 kDa), p2L, p3 and NC (7 kDa) proteins, which are structural proteins (Battles et al., 1992). The Env precursor polyprotein (102 kDa) is cleaved to produce the SU (62 kDa) and TM (40 kDa) glycoprotein that are important in virus-host interaction and entry of virus into the host cell (Gonda, 1992). The Pol precursor polyprotein (120 kDa) is cleaved into a viral integrase, reverse transcriptase and a protease, which are functional enzymes required for replication. The proteins encoded by the accessory genes are Tat (14 kDa), Rev (23 kDa), Tmx (19 kDa) and Vif (predicted size 23 kDa). BIV potentially encodes 2 extra and unique proteins, Vpw (predicted size 7 kDa) and Vpy (predicted size 10 kDa) which have unknown biological function (Gonda et al., 1994). Generally, most of the research on BIV has utilized the original R29 strain (Straub and Levy, 1999; Van Der Maaten et al., 1972) or its derivatives but now more studies are focusing on other isolates of the virus to determine whether R29 infection is a reliable model for BIV infection (Baron et al., 1997).

Pathogenesis of BIV infection

Experimental infections with BIV suggest that in most cattle there is often a mild or sub-clinical response to infection (Flaming et al., 1993). Some early reports of the

effects of BIV infection in cattle utilized BIV contaminated with *Bovine viral diarrhoea virus* which confounded the results obtained. However, it seems that infection can result in transient leucocytosis, lymphoid hyperplasia and lymphadenopathy but unknown longer-term consequences have been suggested (Carpenter et al., 1992). Other related clinical manifestations of BIV infection that have been reported are increased co-infections with *Bovine leukaemia virus* (BLV), hepatic disease, mastitis, decreased immune competence (Zhang and Barkalis, 1997) and other secondary infections, all of which might cause significant production loss (Campbell, 1995).

Jembrana disease

History

The initial outbreak of Jembrana disease occurred in Sankaragung, a village within the Jembrana district of the island of Bali in 1964 and it was originally thought to be haemorrhagic septicaemia due to *Pasteurella*. However, attempts at vaccination failed to eradicate or prevent the spread of the disease suggesting a novel pathogen was responsible. Affected cattle were *Bos javanicus* (Bali cattle) a domesticated form of the wild banteng cattle common in Bali. *Bubalus bubalis* (buffalo) were also said to be involved, although the involvement of buffalo has never been reported again. Within 12 months, the disease was reported in all 8 districts of the island in association with a high case fatality rate (Soeharsono and Temadja, 1995).

The first tissue and blood samples of the affected cattle were submitted in April 1965 to the Bogor Animal Disease Research Institute (ADRI) in West Java, where analysis of these and other samples from field investigations led the Bogor ADRI to conclude that the pathological agent was either rinderpest virus or a rinderpest-like virus. A mass vaccination program against rinderpest was initiated on Bali and (although rinderpest was not the cause) the disease was not reported again for another 7 years when a smaller more localized outbreak was recognized in the Tabanan region of Bali in 1972 (Soeharsono and Temadja, 1995). A third outbreak of the disease was reported in 1981 in the Karangasem region of Bali (Soeharsono, 1995). The disease has also since been reported in Bali cattle in other areas of Indonesia including Lampung Province in Sumatra island (Prabowo, 1995), South Sumatra in 1976 and West Sumatra in 1992 (Tembok and Erinaldi, 1995), and East Java in 1978 (Tembok

and Erinaldi, 1995). Common to all these outbreaks was the movements of Bali cattle from regions where the disease was endemic, suggesting that the infectious agent originated in Bali, and was disseminated in association with the illegal transport of infected cattle from Bali. The disease has since been detected in Kalimantan (Wilcox, 1995) on the island of Borneo but there is no evidence of how the virus was transmitted to Kalimantan.

The aetiological agent was identified as a lentivirus closely related to BIV (Chadwick et al., 1995b; Kertayadnya et al., 1993). Contention exists as to the origin of JDV and 3 main explanations for its origin have been proposed. The first was that in 1964, there was a foreign cattle ship in Buleleng harbour, and although this was 100 kilometres from the site of the first infection it was possibly responsible for the initial outbreak of Jembrana disease in local Bali cattle. The second explanation was that at the time of the initial outbreak, there were mass vaccinations against foot and mouth disease in Indonesia using inactivated virus vaccines and the vaccines might have been contaminated with JDV; this is unlikely considering the instability of lentiviruses and JDV. The final theory, the most plausible, was that banteng, buffalo or deer from the West Bali National Park adjacent to the Jembrana district were the primary host and transmitted the virus to nearby domesticated cattle (Soeharsono et al., 1995c; Soeharsono et al., 1995d).

Host species

The major host cattle species for JDV is now domesticated Banteng (*Bos javanicus*) (Figure 2.4). Studies with other species of cattle such as Friesians (*Bos taurus*), Ongole (*Bos indicus*), buffalo (*Bubalus bubalis*) and cross-bred Rambon and Madura breeds (*Bos javanicus* x *Bos indicus*) have shown that they can be experimentally infected with JDV (Soeharsono et al., 1995d) but the clinical disease and pathological lesions resulting from infection are less severe and the infection in these species could be easily missed under field conditions (Soeharsono et al., 1995b). Sheep, goats and pigs can be infected with JDV and while some pigs developed a fluctuating febrile reaction the only species to develop a detectable viraemia was sheep (Soeharsono et al., 1995a). This suggests that like BIV, the virus associated with Jembrana disease has a larger host range than other lentiviruses, and supports the theory that the original outbreak could have originated from another ruminant species in the nearby national park (Soeharsono et al., 1995a).

Figure 2.4. Female *Bos javanicus* cattle on Bali Island. The coat colour of the cattle is sex-linked: females are light brown as shown and mature males are dark, almost black. Note the distinctive white markings on the rump. Photograph courtesy of Professor Joe Brownlie, Royal Veterinary College.

Jembrana disease virus

The virion is a roughly circular to ovoid enveloped virus of 90-130 nm diameter which uses C-type budding to release from infected host cells (Kertayadnya et al., 1993). The JDV genome is 7,732 bases in length, 750 bases shorter than the BIV 127 genome, an infectious BIV clone derived from the R29 strain by Garvey *et al.* (1990); however, the 2 genomes share 72% nucleotide identity. The major differences between BIV and the JDV genome are deletions of up to 431 bases in length in the SU region of the genome and numerous insertions of up to 33 bases throughout the genome (Figure 2.5). Other differences between the JDV and BIV genomes include several differences in the *cis*-acting sequences (promoters, enhancers, TAR, splice sites and frame shift sequences) and nucleotide changes that could lead to amino acid changes in the coding regions, especially in the *env* regions but also in *gag* and *pol* genes (Chadwick, 1995; Gonda et al., 1994). The JDV genome also lacks the Vpw, p2L, p3 and Vpy proteins found in BIV and there are also probable differences in the processing of the *gag* gene that differ to JDV (Chadwick, 1995).

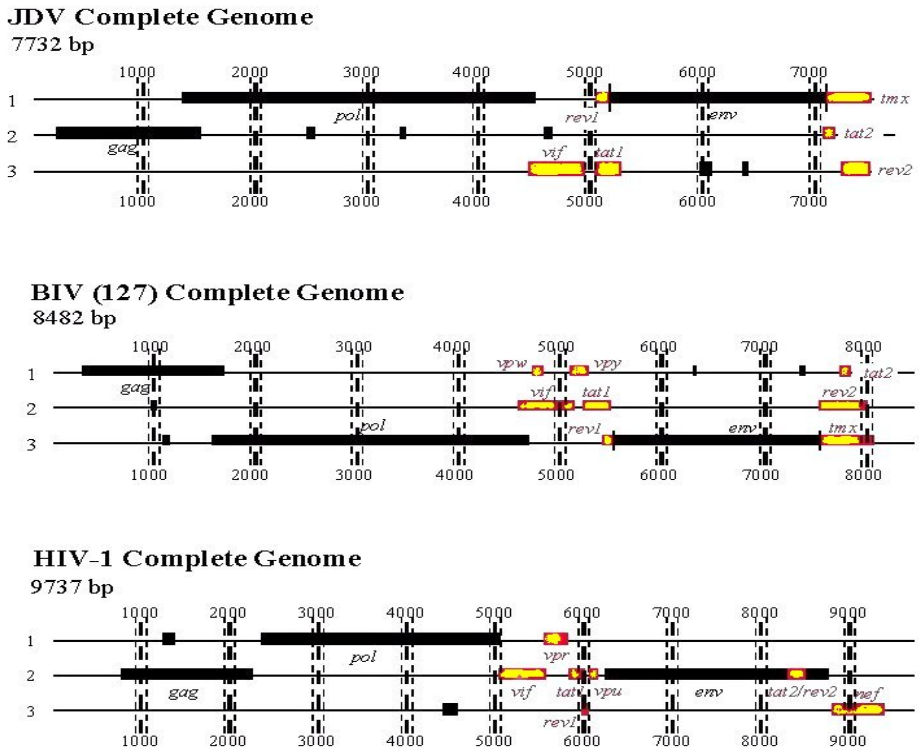


Figure 2.5. Comparative genomic maps of the JDV genome (top), BIV genome (middle) and HIV-1 genome (bottom). The major ORFs are shown in black and the location of the accessory genes is indicated in red and yellow. From Stewart (2005).

Transmission of Jembrana disease virus infection

The mechanism of transmission of JDV has not been determined but probable routes of infection have been identified. One hypothesized method of transmission was from cattle during the acute phase of infection when circulating virus levels reach 10^8 virus particles/mL in blood, suggesting mechanical transmission either by contaminated syringes or by arthropod vectors would be possible (Soeharsono et al., 1995d). Supporting this theory was data indicating that infection seems to require close contact between infected and susceptible cattle, as the spread of infection to cattle on islands close to Bali has not occurred. This indicated that the transmission was either by animal-to-animal contact or mechanically by short-range arthropod vectors that cannot travel large distances. In experimentally infected cattle kept in close contact with each other, transmission from infected to in-contact cattle has been clearly demonstrated (Soeharsono et al., 1990). The possibility of other means of

transmission, such as vertical transmission and body fluids (milk, semen or saliva) have not been investigated fully, but there is evidence that during the acute phase of infection, JDV is shed into milk from infected cattle and that conjunctival and oral infection is possible (Soeharsono et al., 1995d).

Seroprevalence and epidemiology of Jembrana disease

The seroprevalence of JDV has been investigated in Lampung, Sumatra, Kalimantan, Lombok, Sulawesi and Java using sucrose-purified whole virus antigen in an ELISA (Hartaningsih et al., 1993) and the islands where JDV infection has been detected serologically and Jembrana disease has been detected clinically and pathologically are shown in Figure 2.6. The seroprevalence of JDV antibody-positive cattle on Lampung varied from 0-35% in different areas with a mean of 13% over the whole province. Kalimantan had the highest prevalence of seropositive cattle with a range of 15-76% antibody positive cattle in different areas with a mean of 43%. On Java, only one province was tested and there was 31% antibody positive (Figure 2.6). In Sulawesi and Lombok, antibody-positive cattle were not detected (Soeharsono and Temadja, 1995).

Figure 2.6. Areas of Indonesia where JDV infection has been detected (in red). Antibody positive cattle have also been detected in Sulawesi but Jembrana disease has not been detected, suggesting the presence of a second non-pathogenic bovine lentivirus in cattle in this island.

Seropositive cattle have also been identified in South Sulawesi although no clinical signs or other evidence of Jembrana disease have been reported in cattle in Sulawesi

island, suggesting the presence of a non-pathogenic bovine lentivirus (possibly BIV) (Soeharsono, 1995). The serological studies reported by Hartaningsih *et al*. (1995) did not take into account the possible presence of a second lentivirus in Indonesia that could cross-react with JDV antigens and almost certainly could have resulted in the inclusion of false-positives into the data.

A seroprevalence survey was carried out in Bali over a 12-month period, testing different herd sizes and herd intensities using native antigen ELISA (Soeharsono, 1995). The study found that large intensive farms had a higher seroprevalence than cattle owned by smallholders and as the smallholder herd size increased so did the prevalence of seropositive cattle. The number of seropositive cattle was found to be higher in the wet season which suggested there could be more exposure due to increased insect (tabanid or mosquito) activity (Putra and Sulistyana, 1995), again supporting the theory that blood-sucking insects are a likely source of new infections.

Pathology of Jembrana disease resulting from JDV infection

The pathogenesis of the disease was investigated in 18-month-old Bali cattle that were given an intravenous inoculation of JDV, which resulted in clinical signs of disease occurring 4-12 days post-infection (Soeharsono et al., 1995a). The main clinical features of infection were an elevated body temperature, loss of condition, anorexia, lethargy, enlargement of superficial lymph nodes, bloody diarrhoea and a discharge from the nose. The case fatality rate was 17% and although all infected cattle showed clinical signs of infection they did not necessarily exhibit all the clinical changes (Soeharsono et al., 1995a). Jembrana disease causes marked haematological changes in affected Bali cattle: leucopenia as a result of a lymphopenia, eosinopenia and neutropenia, thrombocytopenia, anaemia, increased blood urea concentrations and diminished total plasma protein concentration; these changes occur principally during the febrile period (Soeharsono et al., 1995a). Gross pathological changes include vascular damage such as mild exudates and haemorrhages, but the most striking changes are lymphadenopathy and splenomegaly. Lymphoid tissues of all organs, particularly in the enlarged lymph nodes and spleen, feature proliferating lymphoblastic cells predominantly throughout parafollicular (T-cell) areas, and atrophy of follicles (B-cell areas). A proliferative lymphoid infiltrate was also found in the parenchyma of most organs, particularly the liver and kidneys and an infiltrate containing proliferative macrophage-like cells was found in the lungs (Dharma *et al*.,

1994). This acute and significant pathology was in stark contrast to the relatively mild pathology post-infection with BIV, even though the 2 virus types are genetically similar. Infection with JDV also appeared to immunosuppress cattle which would make them more susceptible to secondary infection and opportunistic pathogens (Wareing et al., 1999). There is anecdotal evidence of an increased occurrence of secondary infections, including haemorrhagic septicaemia, in cattle with Jembrana disease (Wilcox, Personal Communication). This might be expected from the severe lymphoid depletion persisting for at least 6 weeks post-infection (Dharma, 1995).

Virus infection appears to provide protective immunity and attempts to re-infect 18 cattle recovered from experimentally-induced disease as long as 22 months previously were able to induce a febrile response in only one of the re-infected cattle. This indicated that the immunity is maintained over a long period of time and that a vaccine should be effective in giving long term immunity (Soeharsono et al., 1995a).

Molecular genetics of JDV

JDV theoretically could encode 14 proteins (Chadwick et al., 1995b) some of which are shown in the schematic diagram in Figure 2.2. The major components of the virus are the MA, CA and NC proteins that are cleaved from the Gag precursor polyprotein encoded by the *gag* ORF, typical of lentiviruses (Chadwick et al., 1995b). MA is a 125 amino acid (14.3 kDa) protein located at the N-terminal end of the Gag polyprotein. In other lentiviruses, MA is the most abundant structural virus protein, essential in the late virus infection cycle that is targeted to the plasma membrane and involved in incorporation of viral components into budding virions (Keirnan et al., 1998). The JDV MA has a 60% amino acid identity with the MA of the BIV 127 (BIV127) strain and they cross-react antigenically (Desport et al., 2005). The CA protein is cleaved from the central region of the Gag polyprotein and is predicted to be 226 amino acids long (25.3 kDa) and to share 75% amino acid identity with the CA protein of BIV127. The CA protein is the immunodominant protein in BIV and JDV and the CA proteins of the 2 bovine lentiviruses cross-react antigenically with each other (Desport et al., 2005; Kertayadnya et al., 1995) and with other lentiviruses due to a highly conserved major homology region (MHR) (Chadwick et al., 1995b; Cheevers et al., 1988; Rosati et al., 1999). The NC protein is encoded by a region at the carboxy-terminus of the Gag polyprotein (85 amino acids long) and is predicted to

be 9.2 kDa. This protein in other lentiviruses is important for encapsulating structural RNA of the virus within the infected host cell (Zhang and Barkalis, 1997).

The functional intravirion proteins (enzymes) of JDV are encoded by *pol* and are predicted to be expressed as a 1,027 amino acid and 118 kDa Pol precursor polyprotein, which is cleaved by the host cell proteases into an integrase (IN), a reverse transcriptase (RT) and viral protease (PR). The *pol* gene shows 68% nucleotide identity to the BIV 127 *pol* (Chadwick et al., 1995a) but no analysis of the individual enzyme similarity has been undertaken.

The Env polyprotein of JDV encoded by *env* ORF is predicted to be 781 amino acids long (88.8 kDa) and with 31% identity to the BIV 127 Env precursor polyprotein. This protein undergoes further cleavage by the virus-encoded protease to produce SU and TM glycoproteins, and a putative Tmx protein.

The JDV SU is predicted to be 422 amino acids long (47.8 kDa), which is smaller than the BIV127 SU protein, and there is only a 24% amino acid identity between the JDV and BIV proteins (Chadwick, 1995). The vital role of SU in the first stage of the replication process, combined with its ability to induce a strong neutralising and non-neutralising antibody response, has made it a candidate for vaccines and differential tests due to the presence of many epitopes in variable regions (Ball et al., 1992; Gallaher et al., 1995).

The JDV TM protein is predicted to be 359 amino acids (41.1 kDa) with a 39% amino acid identity to BIV127 TM (Chadwick et al., 1995b; Gallaher et al., 1989). The HIV TM is vital to virus-host binding and interaction and consistent antibody responses have been observed against it, which highlights its potential as an additional vaccine candidate (Gallaher et al., 1989).

There are 6 potential accessory proteins encoded by JDV that have yet to be characterized fully, including Tat, alternative Tat, Tmx, Vif, Rev and alternative Rev that could all be important in virus regulation and immune modulation. Tat is 97 amino acids (10.7 kDa) and shares a 54% amino acid identity with BIV 127 Tat while the alternative Tat is 114 amino acids long (12.5 kDa) (Chen et al., 1999; Liu et al., 1992; Setiyaningsih et al., 2008). The main functions of Tat in HIV are to activate transcription of proviral DNA for viral replication, recruit new viral targets (cells bearing CD4+ receptors), shield the virus-infected cell from the immune system and

increase the ability of the infected cells to support viral replication. Detection of Tat antibodies in HIV-infected individuals indicates that this could be an important candidate protein for a vaccine (Pantaleo and Walker, 2000). The JDV Rev is predicted to be 213 amino acids (23.8 kDa) and shows 35% identity to BIV127. The alternate JDV Rev is predicted to be 164 amino acids (22.4 kDa). Rev is theoretically functionally homologous with the cognate HIV proteins and shown in HIV to activate the export of partially spliced and unspliced viral mRNAs throughout the host cell (Pantaleo and Walker, 2000). Vif is a 197 amino acid (22.9 kDa) protein that shows a 55% amino acid identity with BIV127 Vif. The latter stages of viral assembly seems to be affected by this protein but its complete role in virus replication has yet to be elucidated (Miller et al., 2000b). The final JDV accessory protein is the unique Tmx seen only in bovine lentiviruses; this protein is 164 amino acids (18.5 kDa) and has an unknown function (Chadwick, 1995).

Current lentivirus vaccines

There are 4 main strategies that have been successfully used to produce successful vaccines to viral infections: live attenuated virus vaccines, inactivated or killed whole virus vaccines, viral subunit/recombinant protein vaccines, and DNA vaccines. Lentiviral vaccines are extremely problematic as these viruses readily infect immune cells of the host, so priming the immune response by vaccination would recruit cells for the virus to infect.

There is only one commercially available vaccine for any lentivirus and this is for FIV (Uhl et al., 2002). This vaccine is an inactivated whole virus vaccine containing 2 low pathogenic strains of FIV; this protects approximately 2/3 of vaccinated cats depending upon the strain of FIV used to challenge the cats, although in control (non-vaccinated) animals there are also a proportion that are resistant to infection (up to 58% but generally below 20% of the non-vaccinated cats) (Uhl et al., 2002). A major problem with this vaccine is that there is no immunological marker of infection to differentiate vaccinated cats from non-vaccinated cats, which can lead to non-infected vaccinated cats being euthanized (Uhl et al., 2002). This problem, the apparently low level of protection and the cost have prevented the acceptance of this vaccine in many veterinary clinics and animal shelters. These problems demonstrate the difficulty faced when developing a commercial lentiviral vaccine, particularly for animals. Other potential difficulties faced with the development of new veterinary vaccines are

the potential need for maintenance of a cold-chain, potential toxicity and other adverse effects including the presence of endotoxins, the registration process, and the acceptance of the vaccine in the veterinary community (Streefland, 2003).

Live attenuated vaccines

Live attenuated vaccines use viruses that are either naturally occurring or have been altered to reduce the virulence of the organism. The virus is able to infect cells and replicate within the host but is unable to cause disease (Gotch et al., 2000). These vaccines typically, but not always, produce a good immune response in the host with strong antibody and CTL responses and a long-lived immunity to the virus used. This type of vaccine has been used successfully to prevent bacterial infections such as cholera, typhoid, and tuberculosis. They have also been used successfully against viral infections such as hepatitis A, measles, mumps and smallpox in man (Rosen and Mackay, 2001). The acutely pathogenic nature of JDV infection, the inability to culture the virus *in vitro* and the possible reversion to virulence of the virus even if it were attenuated, indicate this type of vaccine is inappropriate for control of Jembrana disease in Indonesia.

Inactivated or killed virus vaccines

These vaccines are derived from whole viruses that have been inactivated, usually by alkylating agents such as formaldehyde. This type of vaccine has been successfully used to prevent mumps, influenza, FIV, poliomyelitis and Jembrana disease (Gotch et al., 2000; Hartaningsih et al., 2001; Hartaningsih et al., 1995a; Mazzetti et al., 1999). These vaccines are not infectious and cannot replicate within the host, adding a degree of safety, and they can often provide adequate protection against infection. Generally there is a good antibody response to the core antigens of the virus but a poor CTL response (Letvin, 2002). Unfortunately, this type of vaccine can lead to an enhancement of infection in some lentivirus infections such as FIV (Hosie et al., 1992). Another common problem with these vaccines is that the chemical agents used to inactivate the virus may remove or structurally alter the outer envelope surface proteins preventing them from being recognized by the immune system which can lead to restricted specificity of neutralizing antibodies, as most neutralizing antibodies target the outer envelope proteins of the virus (Gotch et al., 2000). Early studies with inactivated FIV were promising when a low challenge dose was used and the vaccine

strain was homologous to the challenge virus (Hosie et al., 1995; Yamamoto et al., 1991). However, if a higher vaccine dose was used the results were varied and there was no protection if a heterologous challenge was used (Hosie et al., 1995). The use of FIV vaccine Fel-O-Vax® (Fort Dodge) has provided up to 84% protection against infection with heterologous FIV strains in kittens but the efficacy drops rapidly when a larger challenge dose is used, and there is evidence for enhancement of infection in some cases (Dunham et al., 2006; Matteucci et al., 2000; Uhl et al., 2002).

Recombinant protein vaccines

This type of vaccine is produced by the expression of viral proteins in vector systems such as mammalian, insect, yeast, plant or bacterial cells (Letvin, 2002). Production of virus proteins by these methods is generally easy and low cost but unfortunately these antigens often fail to elicit a CTL or neutralizing antibody response (Letvin, 2002). These vaccines do, however, remove the difficulties associated with the use of attenuated viruses and overcome the problems associated with the use of large scale cell cultures needed for the production of inactivated virus vaccines. In HIV research, a recombinant fused Tat-glutathione-s-transferase (GST) protein vaccine was found to lower plasma virus load and produce an attenuated disease profile to SIV/HIV infection (Pauza et al., 2000). Studies on the use of HIV-1 recombinant Tat-GST as a vaccine have shown that the protein and the tag were internalized and a good antibody and CTL immune response were detected to the antigen (Borsutzky et al., 2006; Tyagi et al., 2001). This approach has also been used for other HIV vaccines with varying degrees of success and a recombinant hepatitis B vaccine is commercially available (Gotch et al., 2000; McElrath et al., 2000; Rusche et al., 1987).

JDV vaccine development

A JDV inactivated virus vaccine was developed using a tissue-derived (spleen) homogenate from an infected donor animal and the addition of Triton-X100 to inactivate the virus (Hartaningsih et al., 1995a; Ramachandran, 1995). This vaccine has been shown to ameliorate the disease and reduce mortality (Hartaningsih et al., 2001; Hartaningsih et al., 1995a). However, it is expensive to produce as approximately 3,000 doses only are prepared from one donor animal. In addition there could be contaminating bacteria or viruses in the donor animal that are able to resist the inactivation process. Inactivation will also probably vary between different

vaccine preparations and there are obvious ethical concerns surrounding infecting and sacrificing increasing numbers of donor cattle (Hartaningsih et al., 1995a).

JDV recombinant protein vaccine candidates

Tat

Tat is potentially encoded for by 2 ORF's, *tat*1 and *tat*2. During HIV infection, multiply spliced *tat* transcripts are produced early in the viral replication cycle before and during the integration phase which leads to the translation of Tat proteins sufficient to initiate transcription of HIV (Fulcher and Jans, 2003). During HIV-1 infection, Tat levels can reach up to 1 ng/mL of serum in chronically infected patients (Albini et al., 1998; Miller et al., 2000a).

In JDV and BIV, there is an in frame stop codon prior to the splice donor on exon 1 that suggests only the *tat*1 is translated during the acute phase of infection (Setiyaningsih et al., 2008). The Tat1 protein of JDV is 97 amino acids with a predicted molecular weight of 10.7 kDa. The predicted protein encoded by JDV *tat*2 is 114 amino acids with a predicted molecular weight of 12.5 kDa and as yet no research has been undertaken to determine the importance and biological role of this second protein. JDV Tat is the most potent of all the characterized lentiviral Tat proteins and can transactivate other lentiviral LTR regions (Chen et al., 1999; Deng et al., 2006).

The remainder of this review of the biological roles of Tat involve HIV-1, unless stated, as this is the best characterized of all the lentiviral Tat proteins.

Structure of Tat

Tat is usually present in monomers and does not form stable multimers in living cells (Rice and Carlotti, 1990; Stauber and Pavlakis, 1998). HIV-1 Tat has 5 conserved regions: an N-terminal, a cysteine rich, a core, a basic and a C-terminal or glycine rich region (Fulcher and Jans, 2003; Jones and Peterlin, 1994; Taube et al., 1999). The Tat family of proteins contain small highly basic stretches rich in arginine and lysine in the N-terminal, core and cysteine rich regions which are involved in RNA recognition, although on its own these regions are not sufficient for RNA recognition (Chen and Varani, 2005; Garber et al., 1998). Another important region of Tat is a 9 amino acid arginine rich region (RKKRRQRRR) in the basic region which allows the

viral protein to penetrate the host cell membrane and translocate to the nucleus (Jeang et al., 1999; Vives et al., 1997). The N-terminal, core and C-terminal regions are important in the structural stability of the protein and the cysteine rich region interacts with cellular proteins involved in transcription elongation (Figure 2.7) (Fulcher and Jans, 2003).

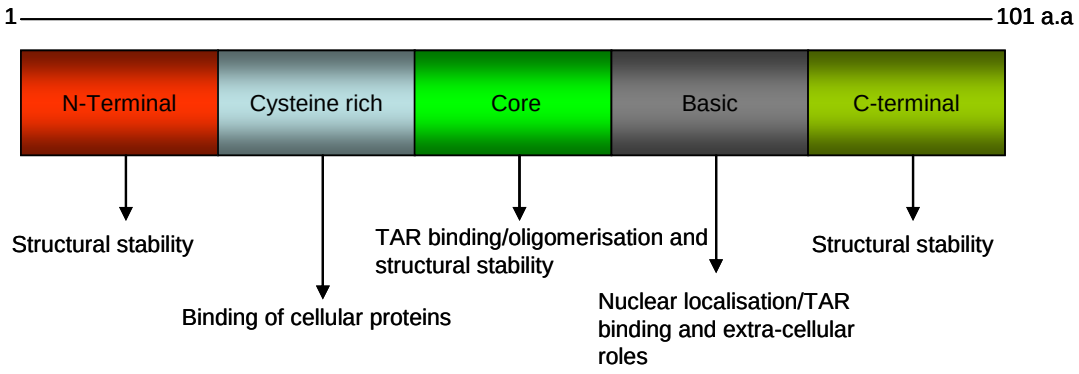


Figure 2.7. Schematic diagram of HIV-1 Tat structure. The 5 structural domains are the N-terminal, cysteine-rich, core, basic and C-terminal domains (Adapted from Fulcher and Jans, 2003).

Intracellular role of Tat

One important feature of all Tat proteins is their ability to translocate through the plasma membranes of cells by matrix-associated heparin sulfate (HS) proteoglycan receptors which allow Tat to penetrate the cell membrane and alter the physiological state of the cell (Chang et al., 1997; Tyagi et al., 2001; Vives et al., 1997). The Tat proteins then localize predominantly in the cytoplasm and they are only transiently present in the nucleus of the host cell even thought they exert effects in the cytoplasm and nucleus (Stauber and Pavlakis, 1998). Once Tat is inside the host cell it can then shuttle between the cytoplasm and nucleus of the host cell.

HIV-1 Tat is essential for late viral replication and its main role is the regulation of transcription which it achieves by activating the viral LTR region to allow efficient transcription of viral genes needed for viral replication (Brady and Kashanchi, 2005; Chen et al., 1999; Jeang et al., 1999; Stauber and Pavlakis, 1998). The Tat protein activates the LTR region by recruiting cellular cyclin T1 and cdk9 which forms the positive transcription elongation factor b (P-TEFb). This complex then binds to *cis* acting RNA enhancer elements (transactivation-responsive region; TAR) leading to

hyperphosphorylation of the C-terminal domain (CTD) of RNA polymerase II and this increases the processing speed of RNA polymerase II (Brady and Kashanchi, 2005) (Figure 2.7). HIV Tat may also induce chromatin remodeling of proviral LTR-mediated gene expression by recruiting histone acetyltransferases that relocate to the chromatin regions leading to histone acetylation (Dandekar et al., 2004).

In JDV and BIV it has been shown that the Tat proteins bind with high affinity and adopt distinct β-hairpin conformations, that when bound to the RNA are sufficient for viral transcription (Chen et al., 1999; Deng et al., 2006; Xie et al., 2003). The Tat of small ruminant lentiviruses such as MVV and CAEV differ from that of primate and bovine lentiviruses as their Tat protein has significantly lower trans-activation activity than the primate and bovine models (Villet et al., 2003).

Other important roles of Tat are its interaction with nuclear factor kappa B (NFκB) enhancer which is an important regulator of numerous cellular genes (Dandekar et al., 2004). Tat has been shown to modulate the expression of many cellular genes such as cytokines, adhesion molecules, oncogenes and MHC class I proteins (Chang et al., 1997). Tat also interacts with many cellular proteins such as TBP, TAFII250, TFIIB with unknown effects (Kashanchi et al., 2000; Marcello et al., 2001). All the biological functions of the Tat protein have yet to be fully elucidated but much of the HIV-1 research on the Tat proteins has shown that this protein is an ideal candidate for a vaccine as it is essential for viral survival and fitness.

Extra cellular role of Tat

HIV-1 Tat has many diverse and poorly understood extra cellular biological roles and only the roles that are most relevant for pathogenesis and vaccination are reviewed.

Extra-cellular Tat induces uninfected CD4+ cells to become more permissive to infection with HIV and is a potent chemo-attractant for susceptible monocytes/monocyte-derived dendritic cells (Albini et al., 1998; Bartz and Emerman, 1999). To achieve this, Tat mimics β-chemokine features which may recruit chemokine receptor expressing monocytes/macrophages to virally infected cells (Figure 2.8) (Albini et al., 1998).

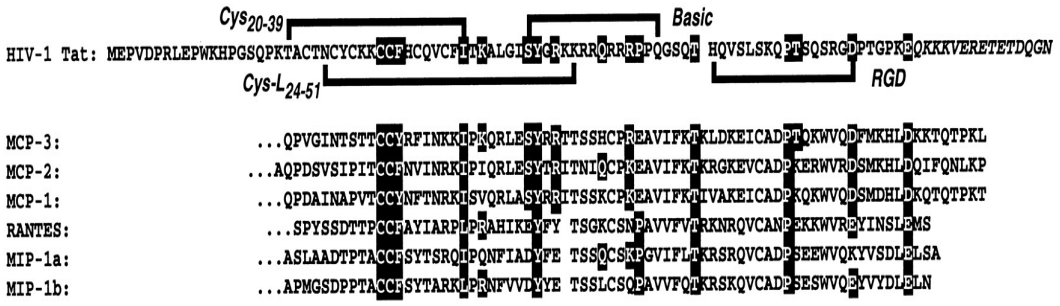


Figure 2.8. Alignment of the HIV-1 Tat protein with the mature peptide sequences of the ß-chemokine MCP-3, MCP-2, MCP-1, RANTES, MIP-1a and MIP-1b. Conserved residues are shaded in black and the location of the Tat peptides in brackets. The residues shown in italics (amino acids 87–101), not present in some HIV isolates, were not included in the $Tat_{1–86}$ synthetic peptide used (adapted from Albini *et. al*., 1998)

Tat also induces monocytes to kill uninfected CD4+ cells through a TNF-related apoptosis-induced ligand (TRAIL)-mediated mechanism (Yang et al., 2003). Other important biological roles of Tat are its involvement in a pro-apoptotic effect on bystander cells and the interaction with viral gp120 to modulate viral entry into the host cell (Giacca, 2005; Marchio et al., 2005). HIV-1 Tat has also been shown to exert angiogenic activity *in vitro* and *in vivo*, and to induce CNS neurotoxicity (Mitola et al., 2000; Rusnati et al., 2000; Tyagi et al., 2001). HIV-1 Tat has also been shown to increase the infectivity of endothelial cells to Kaposi-sarcoma associated herpesvirus (KSHV) by facilitating entry into the host cells (Aoki and Tosato, 2004; Aoki and Tosato, 2007).

Tat vaccines

The extensive intracellular and extra cellular biological roles of Tat and its essential role for viral replication have suggested that Tat is a promising vaccine candidate (Brady and Kashanchi, 2005; Fanales-Belasio et al., 2002; Fulcher and Jans, 2003). The *tat* gene is an early lentiviral regulatory gene and these early genes are generally more conserved than the structural genes transcribed later in viral infection (Stittelaar et al., 2002) and antigenic conservation of the epitopes of Tat would be an important consideration for vaccine development (Caputo et al., 2004).

Tat vaccines have been used in a multitude of different forms, including native Tat, a synthetic peptide, a DNA vaccine, a toxoid and as recombinant proteins (Burgers et al., 2006; Fanales-Belasio et al., 2002; Fulcher and Jans, 2003; Noonan et al., 2003;

Osterhaus et al., 1999; Pauza et al., 2000). Tat has also been used in active and inactive forms. Vaccines that contained biologically active Tat have had substantial side effects such as toxicity, fever, cellular apoptosis and multi-organ lymphoid hyperplasia in the vaccinated animals (Benjouad et al., 1993; Goldstein, 1996; Vellutini et al., 1995; Vellutini et al., 1994). To combat this, many of the vaccines have contained Tat inactivated by chemicals such as hydrogen peroxide or mutated in the active sites of the protein (Noonan et al., 2003).

There are numerous studies showing that vaccination with Tat ameliorates the disease or prevents infection in a proportion of animals (Caputo et al., 2004; Fanales-Belasio et al., 2002; Moreau et al., 2004). It was found that Tat-specific T lymphocyte frequencies were inversely correlated to rapid disease progression (van Baalen et al., 1997) and that Tat neutralizing antibodies provided protection in rhesus monkeys vaccinated with Tat peptides and proteins (Belliard et al., 2005). HIV Tat proteins have also been used in conjunction with Env or Gag proteins and have been found to broaden the T cell responses against the antigens making it a novel adjuvant that may have numerous uses in vaccinology (Gavioli et al., 2008; Gavioli et al., 2004).

Capsid vaccines

Capsid protein lentivirus vaccines have been trialed in human and animals with varying degrees of success. The CA of lentiviruses is the immunodominant protein produced during infection and it contains B and T cell epitopes (Burkala et al., 1998; Hammond et al., 1997; Horton et al., 2001; Whetstone, 1995). However, if used as a vaccine in either a peptide, recombinant protein or whole virus form, little or no protection has been reported and it is, therefore, generally used in conjunction with the Env protein or as a part of the whole Gag antigen or with the accessory proteins (Egan, 2004; Gavioli et al., 2008; Stittelaar et al., 2002). While the Gag antigens can elicit a protective T cell response, studies have shown that the B cell response to these antigens can actually enhance early stage disease similar to what is found with B cell responses directed against certain epitopes on the Env antigen (Chung et al., 2004; Chung et al., 2005; Jolly et al., 1989; Tirado and Yoon, 2003; Torsteinsdottir et al., 2007). There is also evidence that some T cell responses can also enhance infection to certain epitopes on this protein, making the addition of this antigen to vaccine compositions problematic (Nenci et al., 2007). Interestingly, it has been found that

greater protection is provided to cats vaccinated with HIV CA than cats vaccinated with FIV CA and challenged with a homologous strain of FIV (Coleman et al., 2005). This suggests that cross-species vaccination may be more effective than homologous vaccines due to the removal of deleterious functions of the CA vaccine component.

Diagnostic assays for lentiviruses

The development of diagnostic assays for lentiviruses is fraught with difficulties as the viruses infect and compromise the function of immune cells and the potent immuno-modulators like the Tat protein which are produced during infection (Albini et al., 1998; Dandekar et al., 2004; Gavioli et al., 2004). Another problem with diagnosis of lentiviral infections is the window between infection and the development of a serological response, and the undulating antibody response in long term infected animals (Andersson et al., 1997; Pantaleo and Walker, 2000; Saman et al., 1999). Other factors that also affect the sensitivity and specificity of diagnostic tests are population variation, pregnancy status, health status and nutritional status of sub-populations (Greiner and Gardner, 2000). For all these reasons there is no single diagnostic assay for any lentiviral infection that is 100% sensitive or specific, and generally a combination of assays is needed to confirm infection.

Detection of JDV

Detection of JDV is achieved via a number of different techniques, but a low cost-commercial assay with the required degree of sensitivity and specificity is unavailable and is required. JDV infection has been detected using serological tests, particularly ELISA and Western immunoblotting with either native antigens to detect antibodies produced in infected cattle (Hartaningsih et al., 1995b) or recombinant protein antigens (Burkala et al., 1998). The use of native antigen (sucrose gradient purified whole virus present in plasma of infected animals) is an accurate way of detecting the antibodies to the virus but it is not viable for large-scale screening; the production of this antigen requires the infection of cattle and harvest of the virus from plasma of the infected cattle, as efforts to culture JDV have thus far been unsuccessful (Kertayadnya, 1995).

The production of recombinant proteins represents an easy and cost-effective method of producing JDV antigens. Unfortunately, such proteins often lack the sensitivity and specificity of native antigens (Abed and Archambault, 2000), which may be due to the

expression vector chosen or different patterns of post-translational modifications than those that occur in natural infections. Other limitations of recombinant proteins are that they do not have all the antigenic proteins of a virus, and they do not overcome the problem of cross-reactivity with JDV and BIV (Barboni et al., 2001). The use of an ELISA with recombinant protein antigens appears to be appropriate for detection of JDV infections, but problems such as the purification of antigen and cross-reactivity need to be overcome before a testing program can be implemented. Proviral PCR with JDV-specific primers is also used to detect JDV infection but this method, whilst extremely sensitive and specific, is time-intensive, costly, and thus not viable as a large-scale diagnostic test (Stewart et al., 2005).

In situ hybridization (ISH) can also be used to detect JDV in tissue samples and viral RNA is present in a large range of host tissues (Chadwick et al., 1998). While this technique is relatively sensitive and accurate it is unfortunately also time consuming and its use restricted to post-mortem samples (Chadwick et al., 1998).

Antibody response to JDV infection

In cattle experimentally infected with JDV, an ELISA with a whole virus antigen initially detected an antibody response in a low percentage of 19 cattle but it was not until 11 weeks after infection until an antibody response was detected in a majority of cattle, and 23 weeks before all cattle were positive. The antibody persisted until 59 weeks after infection, when the observations were discontinued (Hartaningsih, 1993). Western immunoblot examination of 3 of the infected cattle detected a JDV CA antibody response 6 weeks post-infection and it was longer before antibodies against other virus proteins were detected (Hartaningsih, 1993). Ditcham et al. (2007) used recombinant protein antigens and Western immunoblotting to initially detect antibodies against CA and TM proteins 5-15 weeks after infection in most cattle.

Identification of BIV infection

Identification of BIV infection can be achieved via PCR, real-time PCR (Lew et al., 2003), nested PCR (Suarez et al., 1995), seroconversion to the Gag proteins (CA and/or MA) and TM protein (Scobie et al., 1999), or a combination of all of the above but as yet no method has been able to consistently achieve high sensitivities and specificities (Abed et al., 1999; Zhang et al., 1997b). The lack of a “gold standard” diagnostic test is due to a combination of factors, including the difficulty of detecting

virus in infected animals, the antigenic cross-reactivity of the CA proteins with those of JDV, and the loss of antibody response over time in some infected animals (Orr et al., 2003). The current serological tests for BIV can only determine whether the animal has been infected within the last 2 years and the whole virus agar gel immunodiffusion (AGID) assay was found to be the most sensitive and specific compared to ELISA and an immunofluorescence assay (IFA) for the detection of antibody (Orr et al., 2003). Comparisons between ELISA and Western immunoblotting found that whilst there was generally good agreement between the 2 techniques, immunoblotting was more sensitive but less specific than the ELISA (Bhatia et al., 2008; Zheng et al., 2000). A CA-based competition ELISA had a much higher concordance to immunoblotting than the typical indirect CA antigen ELISA suggesting this may overcome sensitivity issues with the indirect CA ELISA (Bhatia et al., 2008).

Antibody response to BIV

The antibody response of cattle infected with BIV strains R29 and FL112 can be detected earlier than with JDV, as soon as 22 days post-infection, with a high titre of antibodies against the CA (p26) protein appearing first and lasting for at least 30 months post-infection, indicating CA is the immunodominant protein during infection (Battles et al., 1992; Whetstone, 1995). Antibodies to the p110, p55 (SU), p24 (Vif), p18 (Tmx), p15 (MA) and p13 (NC) were also detected within 31 days post-infection. Antibody to the p110 was detected transiently after the initial infection but was detected again 270 days post-infection and was maintained until 16 months post-infection (Whetstone et al., 1990b). Isaacson *et al.* (1995) reported that the p26 antibody response declined 10 months post-infection, which conflicted with a report by Whetstone *et al.* (1990). TM antibodies were detected by (Scobie et al., 1999) approximately 4 weeks post-infection and they remained detectable until 50 weeks post-infection in most cattle, suggesting that detection of TM antibody could be used in conjunction with other markers to produce a reliable serological marker of infection (Scobie et al., 1999).

Because of the differences observed in the antibody response between cattle there is no “gold standard” serological test for BIV but an antibody response to a combination of virus protein antigens provides the best indicator of infection (Abed et al., 1999; Burkala et al., 1998). It is interesting that cattle in a Jembrana disease-free region of

Indonesia (Sulawesi) have been tested using native JDV antigen and found to be seropositive. It is likely, due to the homology between JDV and BIV (Burkala et al., 1998), that this is antibody to a non-pathogenic BIV-like virus, and this antibody could lead to false positive results during immunosurveillance for JDV (Hartaningsih, Personal Communication).

Antigenic cross-reactivity of lentivirus proteins

A major impediment to the specificity of the serological diagnosis of lentiviruses in animals is the cross-reactivity of antigens in different species of lentiviruses, particularly troublesome when different species infect the same host. Grund *et al.* (1994) showed that the EIAV CA protein has cross-reactive epitopes to HIV-1 CA epitopes, Rosati *et al.* (1999) showed that CAEV CA epitopes cross-reacted with MVV CA epitopes, Battles *et al.* (1992) found that BIV CA epitopes cross-reacted antigenically with HIV-1 CA epitopes, and Kertayadnya *et al.* (1993) and Desport *et al*. (2005) demonstrated that the CA proteins of BIV and JDV contained several cross-reactive epitopes. This degree of cross-reactivity makes serological differentiation between lentivirus infections of the same species extremely difficult e.g. JDV and BIV-like lentiviruses in Indonesian cattle. A highly conserved region within the CA protein of lentiviruses, the MHR contains conserved epitopes that cross-react antigenically in all lentiviruses. There appears to be a greater degree of specificity in the proteins encoded by the *pol* and *env* genes but recent evidence has shown that conserved epitopes on the surface envelope protein of lentiviruses are also present on the surface envelope proteins of other retroviruses and may lead to serological cross-reactivity with these viruses (Hotzel and Cheevers, 2000).

Chapter 3. Development of a recombinant protein antigen for the serological diagnosis of Jembrana disease virus infection

Summary

Several different hexahistidine tagged recombinant CA and TM proteins were produced and tested as antigens for the detection of antibody against Jembrana disease virus (JDV) in cattle, as alternatives to the current reliance on a whole virus antigen prepared from tissues of infected cattle. A recombinant CA protein used in an ELISA provided the greatest concordance (98.5%) with the whole virus antigen immunoassay and a high sensitivity and specificity made it an ideal and relatively inexpensive replacement for the whole virus antigen. Other recombinant protein antigens produced were not suitable for use, including a fused CA/TM protein antigen which when used in ELISA had high specificity but a low sensitivity, and a fused CA/TM protein that when used in Western immunoblots provided low concordance with results obtained with the whole virus antigen.

Introduction

Diagnosis of lentivirus infections by serological methods, such as ELISA and Western immunoblotting, has typically been difficult (Hecht et al., 2002; Janssen et al., 1998) as the viruses principally infect cells of the immune system, including macrophages, B-cells and T-cells, leading to a compromised immune system (Levy, 1993d). Lentiviruses have many mechanisms for evading immune responses or killing immune effector cells which can further interfere with serological diagnosis (Bartz and Emerman, 1999; Chien et al., 2004; Kerkau et al., 1997). The initial phase of infection presents a special problem for the diagnosis of all lentivirus infections as there is often an extended period where the animal is infected with virus but there is no detectable serological evidence for infection (Houwers and Nauta, 1989; Janssen et al., 1998). Following this initial phase, most but not all animals seroconvert to the CA, followed by seroconversion to other virus proteins as the antibody response matures over time (Battles et al., 1992; Cole et al., 1998; Straub and Levy, 1999). Other factors that further complicate the serological detection of lentiviruses are that the antibody response against the CA antigen cycles over time and has been shown to become undetectable after a prolonged period in some animals (Hammond et al., 1997; Isaacson et al., 1995b).

To overcome these difficulties with regard to the reliable detection of antibody, various strategies have been implemented such as using a combination of peptides

representing epitopes from more than one viral antigen, using a combination of different whole antigens, and developing constructs that express the CA protein with a TM peptide attached for extra specificity (Hodinka et al., 1998; Rosati et al., 2004). JDV has been routinely diagnosed by indirect ELISA and Western immunoblot using whole virus antigens prepared from plasma of infected cattle and purified by sucrose gradient centrifugation (Hartaningsih et al., 1995b). The limitations of this assay include the need to harvest the virus from infected cattle, which creates ethical problems and would also inevitably lead to considerable variation in quantity and quality of the antigen preparations from different animals, the need for expensive equipment for purification of the virus, and the cost. Alternative recombinant GST fusion protein antigens for JDV were initially developed by Burkala et al. (1998) and more recently a recombinant biotinylated CA antigen expressed in the Pinpoint (Promega) system was tested by (Desport et al., 2005). Unfortunately, the assays developed had poor concordance when compared to the whole virus assay making the production of a reliable means of detecting JDV infection a priority for research.

This Chapter describes the development of different systems for the expression of the CA and TM proteins as antigens for ELISA and Western immunoblot. The results obtained with these antigens were compared to those obtained with Western immunoblot with a whole virus antigen.

Materials and methods

Primers and cloning

Plasmid DNA from a JDV clone Jgag6 (Desport et al., 2005) containing the entire JDV CA derived from JDV_{Tab87} was used as template for production of a fused JDV CA/TM polyprotein similar to studies with other lentiviruses (Bertoni et al., 1994; Dufour et al., 2002; Pancino et al., 1995; Soutullo et al., 2001). Amplification was performed using primers jCABamF and jCATmEcoR (Table 3.1) and the amplified

product was digested with appropriate enzymes before ligation into pTrcHisA plasmid digested with BamHI and EcoRI. Plasmids were transformed into competent Top 10F' *Escherichia coli* and the resulting construct, containing JDV CA sequence (nt 604 – 1222 of JDV_{Tab87}) fused directly to the putative TM principal immunodominant domain epitope, was confirmed by direct sequencing of the PCR products obtained using the pTrcHisF and pTrcHisR primers. The plasmid was also transformed into *E. coli* BL21 for expression of JDV CA/TM. The entire JDV *capsid* gene (nt 604-1222 of JDV_{Tab87}) was digested out using BamH1 from purified JDV clone CAΔTat (Chapter 4) and ligated into a pTrcHisA plasmid digested with BamH1 (jCApTrcHisA). Amplification of a second PCR product containing the truncated *tm* gene (6688-6742 of JDV_{Tab87}) (Chadwick, 1995) using the jTmBgIIIF and the jTmEcoR primers (Table 3.1) was performed and the PCR product ligated into the jCApTrcHisA digested with EcoR1 and BgIII. Plasmids were transformed into competent Top 10F' *E. coli* and cloning was confirmed by sequence analysis. The plasmid was then transformed into *E. coli* JM109 for expression of the protein JDV CAΔTM.

Table 3.1. Primer sequences used for the amplification, sequencing and development of recombinant plasmid constructs used for the production of recombinant proteins.

Primer	Sequence (5′ → 3′)	Genome	Nucleotide position in JDV_{Tab87} genome
jCABamF	GGACGGatccCCACAACTTAGAAAGAACTTC	JDV	604-627
jCA/TMEcoR	CCTTTAGGGCCCTCAGCC/CCGGTACAAACT GGGCTAGGGTGTGTGCCGAGAGGAAGATAT TGCCATTTTGATGaattc	JDV	1222-1205 6688-6742
jTmBgIIIF	GATCATTAgatctCAGCCGTGGGGATGGTCATA	JDV	6464-6483
jTmEcoR	TCAGTCAAAATGGCAATAGaattc	JDV	6742-6725
pTrcHisF	GAGGTATATATTAATGTATCG	pTrcHis	370-390
pTrcHisR	GATTTAATCTGTATCAGG	pTrcHis	614-597

Production of hexahistidine-fused recombinant viral proteins

Three constructs were prepared: JDV CA containing the entire theoretical JDV *capsid* gene in a pTrcHisC plasmid, CAΔTM containing the full length JDV *capsid* gene and an 87 a.a. carboxy terminus truncated *tm* gene in a pTrcHisA plasmid. A third construct CA/TM containing the entire *capsid* gene fused to the principal immunodominant domain of the *tm* gene in a pTrcHisC plasmid was also developed (as described above). Recombinant JDV CA was produced from a recombinant pTrcHisC plasmid kindly supplied by Margaret Collins (Royal Veterinary College) containing the entire JDV *capsid* gene (Barboni et al., 2001). This plasmid was transformed into *E. coli* (JM109) for protein expression. The optimum expression of the CA was obtained using 2YT plus 1 mM ampicillin inoculated with a 1:25 volume of an overnight culture, and induced at an OD_{600} of 1.0 by the addition of 0.1 mM IPTG for 6 h. The optimum expression of the CAΔTM was obtained using 2YT plus 1 mM ampicillin with a 1:25 inoculum of overnight culture induced at an OD_{600} of 1.0 with 0.1 mM IPTG for 4 h. After induction, pelleted bacterial cells were resuspended in cell lysis buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM imidazole and 5% [v/v] glycerol) and lysed by sonication (8 pulses of 30 seconds with 30 seconds rest between pulses) and the lysate was centrifuged (10,000 g, 15 min). The pellets were discarded and 10 volumes of lysate were added to 1 bed volume of Ni-NTA agarose resin (QIAGEN) in a chromatography column (BioRad) and incubated at 4ºC for 2 h on a slow rotating mixer. These lysates were allowed to flow through the resin and 4 wash steps were performed using native wash buffer pH 8 (250 mM NaH_2PO_4, 2.5 M NaCl and 50 mM imidazole). Four bed volumes of elution buffer pH 8 (250 mM NaH_2PO_4, 2.5 M NaCl and 250 mM imidazole) were then added to the column and collected. The purified antigens were analysed by SDS-PAGE and Coomassie Brilliant Blue staining to determine yield and purity. Western immunoblotting was also performed to confirm that the proteins were recognized by sera from experimentally infected cattle.

Detection of hexahistidine tagged antigens

SDS-PAGE gels were immobilized on a nitrocellulose membrane using a Mini Protean II transfer tank (BioRad) in transfer buffer (192 mM glycine, 25 mM Tris and 20% [v/v] methanol) overnight at 30 V at 4°C. Once removed from the transfer tank the nitrocellulose membrane was stained using Ponceau stain and placed in a skimmed milk blocking solution (Diplomat skimmed milk powder 5% [w/v] in PBS/T) for 30 min on a horizontal rocker. The blocking solution was then removed and the membrane incubated in skimmed milk blocking solution containing a 1:2,000 dilution of mouse anti-hexahistidine monoclonal antibody (ICN) for 1 h with gentle agitation at room temperature. The membranes were washed 3 times for 5 min with PBS/T and a 1:2,000 dilution of HRP cojugated goat anti-mouse IgG (ICN) in skimmed milk blocking solution for 1 h with gentle agitation at room temperature, followed by an additional 3 washes with PBS. The reaction was then developed using a horseradish peroxidase (HRP) substrate solution (BioRad HRP substrate 10 mg, 16.6 mL PBS pH 7.4, 3.325 mL methanol and 10 µl of hydrogen peroxide) and the strips developed in the dark for 1 h at room temperature. The reaction was then stopped by washing with de-ionized water.

Quantification of protein concentration

Densitometry was used to quantify the concentration of protein in the vaccine preparations and diagnostic reagents. Appropriate dilutions of each preparation were subjected to SDS-PAGE (Laemmli, 1970) in a MiniProtean system (BioRad) with 12.5% polyacrylamide gels, and the resulting gels stained with Coomassie Brilliant Blue. A range of concentrations between 125 ng and 1 µg/lane of standard proteins, bovine serum albumin (BSA) (66 kDa) and lysozyme (16 kDa), were run on each gel. After de-staining, washing and re-hydrating in distilled water, the gels were imaged on a ProExpress Imaging System (PerkinElmer), and the resulting high resolution images subjected to image analysis with ProPick software (PerkinElmer). Pixel density mapping was used to calculate the amounts of protein present in each non-saturated band on the imaged gel. The amount of protein present was then deduced from the standard curves derived from the bands of known amounts of the standard proteins, lysozyme and BSA.

Whole virus antigens

A range of whole virus antigen preparations supplied by Dr Nining Hartaningsih (DIC Denpasar) were tested by SDS-PAGE analysis and Western immunoblotting and the best preparations were selected and pooled for use as the "gold standard" to compare the diagnostic antigens to determine sensitivity and specificity.

SDS polyacrylamide gels (SDS-PAGE)

The proteins of interest were visualized by SDS-PAGE using a resolving gel (12.5% [w/v]) and a stacking gel (4% [w/v]) in a Mini Protean gel system (BioRad) containing 0.1% SDS [w/v] as described by (Laemmli, 1970) for 54 min at 200 V with a running buffer containing 25 mM Tris, 192 mM glycine and 1% [w/v] SDS. Precision Plus (BioRad) 100 kDa molecular weight markers (5 µL per lane) were added in the marker lane and the protein of interest (diluted 1:1 in 2X loading buffer) was loaded into a single large well. Gels were removed and either stained with Coomassie Brilliant Blue or blotted onto nitrocellulose. Briefly gels were added to 50 mL of Coomassie Brilliant Blue stain (45% [v/v] methanol, 45% [v/v] distilled water, 10% [v/v] glacial acetic acid and 0.05% [v/v] Coomassie Brilliant Blue) were stained overnight at room temperature on a horizontal rocker. Once stained, the excess stain was removed using a de-stain solution (40% [v/v] methanol, 50% [v/v] distilled water and 10% [v/v] glacial acetic acid). This de-stain solution was replaced at least twice until the gel was fully de-stained. A gel drying solution was then added (20% [v/v] ethanol, 3% [v/v] glycerol and 77% [v/v] distilled water) to the gel and left for 30 min. The de-stained gels were then dried between cellulose sheets and stored.

Serum samples

Thirty serum samples from cattle sourced from the Jembrana disease-free island of Nusa Penida (Hartaningsih et al., 1995b) were used as reference negative sera for establishing a cut-off value between negative and positive samples; these samples did not react in Western immunoblots with whole virus antigen.

Ten serum samples from cattle experimentally infected with JDV (Soeharsono et al., 1990) and with virus-specific antibody confirmed by whole-virus Western immunoblotting were used as positive reference sera.

Serum from a well-characterized long-term multiply infected heifer (A4) was used as the positive control for all ELISA and Western immunoblots. A second positive control was taken from a hyper-immunized heifer (A577) and was used for Western immunoblotting to detect responses against all viral proteins. This animal was vaccinated weekly for a 5-week period with inactivated virus as described by Hartaningsih et al. (2001).

Two hundred additional cattle sera were obtained from adult cattle in the Tabanan region of Bali, a Jembrana disease-endemic area. These 200 sera were tested by Western immunoblot with whole virus antigen (Hartaningsih et al., 1995b) to determine the presence of antibody against JDV, and also examined for reactivity against the recombinant protein antigens.

Development of ELISA using recombinant proteins

All recombinant proteins were tested by ELISA using 50 µL volumes of reagents except in the blocking step where a 100 µL volume was added to the wells. The ELISA plates used were Maxisorb (NUNC) and the standard blocking solution used for both the ELISA and the Western immunoblot was a 5% solution of skimmed milk powder (Diplomat) in PBS/Tween 20 (0.05% [v/v]). Wells were coated with 75 ng (2 rows), 50 ng (2 rows), 25 ng (2 rows) and 10 ng (2 rows) of protein in 0.1 M carbonate buffer pH 9.5 (160 mL of 0.2 M Na_2CO_3 and 340 mL of 0.2 M $NaHCO_3$) and the plates incubated overnight at 4°C. The plates were then washed once with PBS/T, and blocking solution was added for 30 min at room temperature. The blocking solution was then removed and the plates rinsed twice with PBS/T. Serial dilutions of the reference negative and positive sera in PBS/T with 5% skimmed milk were added to the appropriate wells on the ELISA plate. ELISA plates were incubated for 1 h at 37°C, and then washed 3 times with PBS/T. A 1:2,000 dilution of rabbit anti-bovine IgG conjugated to HRP (secondary antibody) in PBS/T (MPBio) was added and then incubated for 1 h at 37°C. The plates were then washed twice with PBS/T and twice with PBS (pH 7.4). HRP colour substrate reagent (BioRad) was added to the wells and the reaction developed for 15 min. The reaction was stopped by the addition of 2% [w/v] oxalic acid and the absorbance determined at OD_{405}. The optimum quantity of protein was assessed by the highest signal to noise ratio (mean of the positive reference serum/mean of the negative reference serum).

A high background noise to signal ratio was observed when PBS/T only was used as the blocking agent and various alternate blocking solutions containing 1% casein and 1-5% skimmed milk solutions were tested on plates that were incubated with the optimum amount of protein per well and rinsed once with PBS/T. The reactions were completed as described above and the optimum blocking solution and diluent was chosen for each protein according to the difference in absorbance between the negative and positive samples at OD_{405}. The absorbance of the proteins was read 5, 10 and 15 min after the addition of substrate with the optimal time being chosen according to the development of substrate (OD_{405} of 2-3 with the positive controls).

Determining ELISA cut-off values and statistical analysis

ELISA plates were incubated overnight with an optimum quantity of protein and blocked with the optimum blocking solution. The plates were then rinsed with TBS/T and 1: 200 dilutions of each serum (the 30 reference negative samples and 10 reference positive samples) were added in duplicate to the wells of the ELISA plate and incubated at 37°C for 1 h. The assay was then completed as described above and the OD_{405} of the negative samples was recorded. A mean absorbance of the negative sera was calculated and then a cut-off value for a negative/positive value was determined as the mean absorbance of the negative sera plus 3 standard deviations (SD) of the mean. User-defined two-graph receiver operating characteristic (TG-ROC) (Greiner et al., 1995) was used with a Microsoft EXCEL (version 2003) spreadsheet to validate the cut-off values for the different antigens using known positive (n=10) and negative (n=30) reference serum. TG-ROC analysis plots the sensitivity and specificity against the selected cut-off value assuming the latter to be an independent variable. This generated a range in which the samples are deemed borderline with a pre-selected accuracy level of 95% sensitivity and selectivity. The agreement between the ELISA and Western immunoblot assays was assessed by concordance (percentage overall test agreement) and by kappa values (to test that the observed agreement is beyond chance agreement). Kappa values can vary from 0.0 to 1.0, with values above 0.75 representing excellent agreements beyond chance. Values ranging from 0.4 to 0.75 indicated a fair to good agreement beyond chance whilst values below 0.4 represented poor agreement beyond chance and 0.0 indicated that the agreement was entirely attributable to chance (Fleiss, 1981).

In routine assays, the cut-off values were assessed in relation to a known positive reference sample (A4) and a known negative reference sample (CB15) which were run on ELISA plates in duplicate to standardize the plate to plate differences. If the known positive and negative reference serum were within an acceptable range (2 SD), (OD_{405}1.62-1.98 and OD_{405} 0.18-0.22) then the results were considered valid.

Western immunoblotting

Western immunoblotting was performed as described above. Recombinant proteins were loaded (2,000 ng/well) into an SDS-PAGE gel. Electrophoresis was performed using the minigel sytem described previously and the gel was then transferred onto a nitrocellulose membrane as described by (Towbin et al., 1979) in ice cold transfer buffer overnight at 30 V at 4°C. The nitrocellulose membrane was then placed in 10 mL of Ponceau stain for 30 sec to stain the transferred protein bands. The membrane was then rinsed with de-ionized water and incubated at room temperature for 1 h in blocking solution (5% skimmed milk powder in PBS/T). After removal of the blocking solution, the membrane was cut into strips; each strip was used to test individual serum samples. The appropriate dilution of serum to be tested (either 1:25 or 1:50) was added and gently agitated for 1 h at room temperature. Individual strips were then washed 3 times with PBS/T for 5 min on a horizontal rocker at room temperature. Rabbit anti-bovine IgG HRP-conjugated (1:2,000 dilutions in 5% skimmed milk powder blocking solution) was then added and agitated gently for 1 h at room temperature. The strips were washed 3 times with PBS pH 7.4 before immersing in substrate solution (BioRad substrate HRP 10 mg, 16.6 mL PBS pH 7.4, 3.325 mL methanol and 10 µL of hydrogen peroxide) and the strips developed in the dark for 1 h at room temperature. These strips were then lined up with the protein marker and fixed to a cardboard block for comparison and identification of antibody response to the protein band of interest.

Results

Optimisation of growth of constructs and induction times for expression of hexahistidine tagged proteins

2YT was selected as the growth medium and a the bacterial growth curves were assessed for each of the 3 different constructs by comparing inoculation with 3 different dilutions of overnight culture over an 8 h period. The growth of the constructs containing JDV CA and CA/TM (1:25 dilution of overnight inoculum) started to plateau 5 h post-inoculation and thus a 4 h post-inoculation at an OD_{600} of 1.5 was selected as the optimum for induction of protein expression (Figure 3.1). Expression of JDV CA and CA/TM was detected until 24 h post-induction with no significant protein degradation and an induction time of 12 h was implemented as this gave a similar yield to a 24 h post-induction period. However the fused JDV CAΔTM protein started to degrade 2 h post-induction and was completely degraded 4 h post-induction (Figure 3.2). The CA and the CA/TM peptide fused proteins were of sufficient purity and concentration for use in a recombinant protein ELISA (Figure 3.3) but the CAΔTM polyprotein yield and purity was insufficient for ELISA and the protein was used for Western immunoblotting only. The best yield of purified CA recombinant protein obtained was 46.3 mg/L of culture with purity greater than 95%. The best yield of CA/TM recombinant protein was 5.6 mg/L with 90% purity.

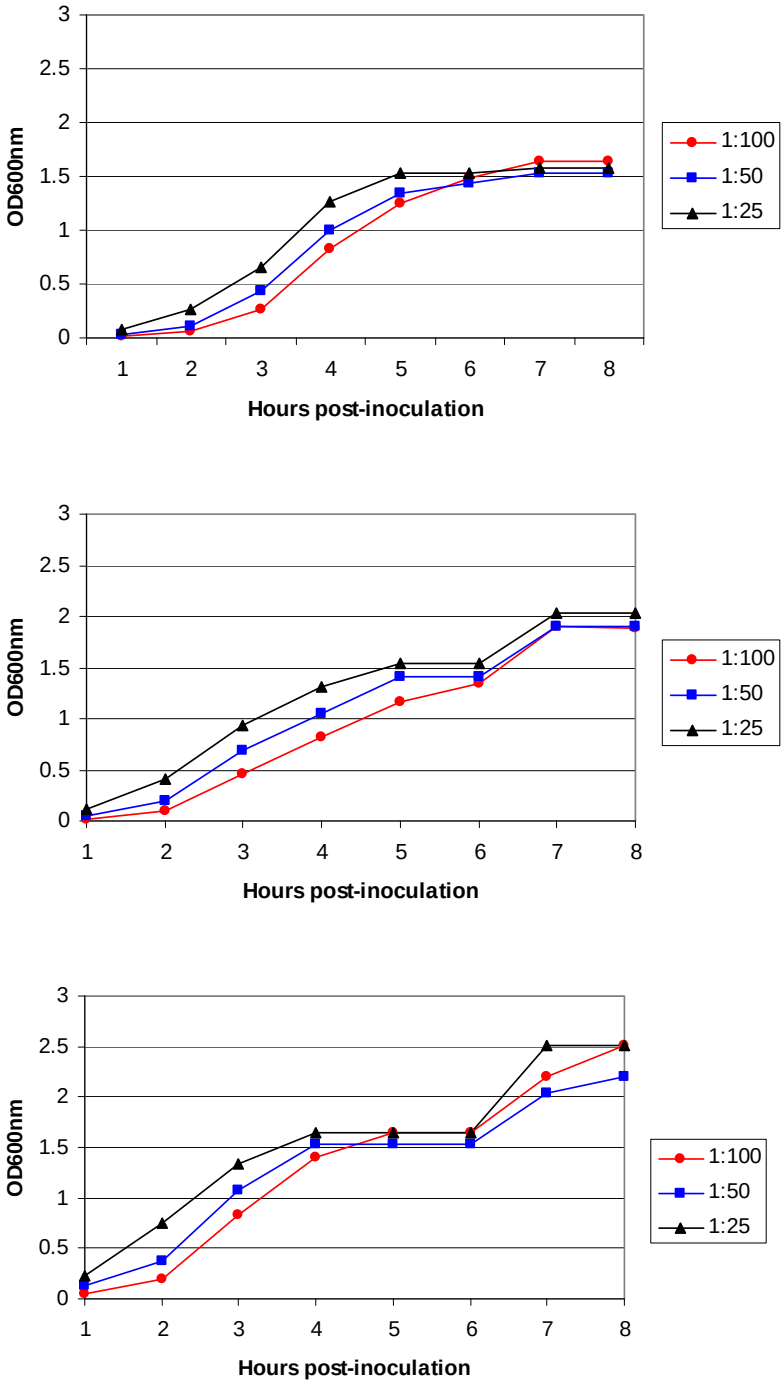


Figure 3.1. Growth curves of the CA, CA/TM and CAΔTM constructs used in the development of a serological antigen for JDV; the constructs were seeded into the medium using a 1:100, 1:50 or 1:25 dilution of the overnight seeding culture to determine the optimum dilution for enhanced bacterial growth. (Top) JDV CA construct in *E. coli* BL21 grown in 2YT medium plus ampicillin, (Middle) JDV CA/TM construct in *E. coli* BL21 grown in 2YT medium plus ampicillin; (Bottom) JDV CAΔTM construct in *E. coli* BL21 grown in 2YT medium plus ampicillin.

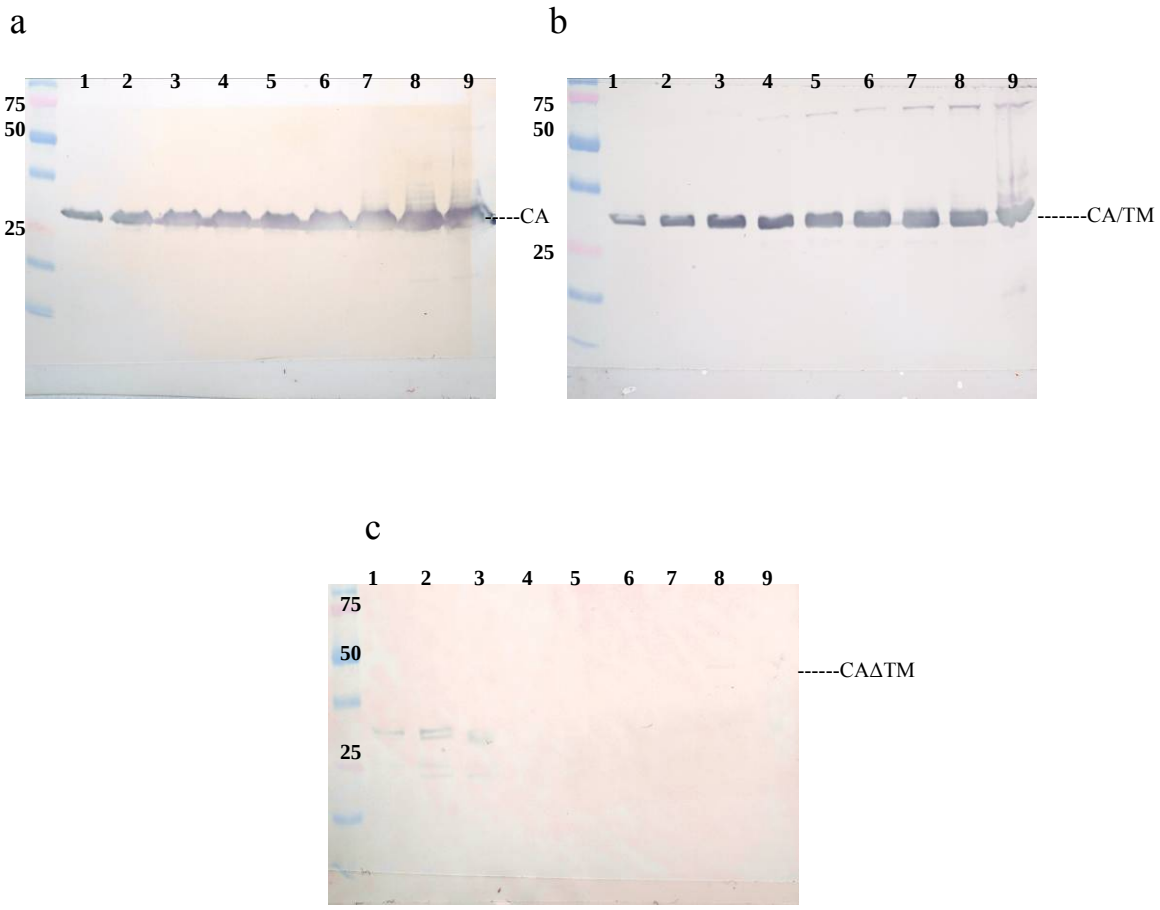


Figure 3.2. Western blots to determine optimum expression time and relative level of protein expression from the CA, CA/TM and CAΔTM constructs. (a) JDV CA construct in E. *coli* BL21 grown in 2YT media plus ampicillin induced for up to 24 h; lane 1, 1 h; lane 2, 2 h; lane 3, 3 h; lane 4, 4 h; lane 5, 5 h; lane 6, 6 h; lane 7, 12 h; lane 8, 18 h; lane 9, 24 h. (b) JDV CA/TM construct in *E. coli* BL21 grown in 2YT media plus ampicillin induced for periods up to 24 h: lane 1, 1 h; lane 2, 2 h; lane 3, 3 h; lane 4, 4 h; lane 5, 5 h; lane 6, 6 h; lane 7, 12 h; lane 8, 18 h; lane 9, 24 h. (c) JDV CAΔTM construct in *E. coli* BL21 grown in 2YT media plus ampicillin induced for periods up to 24 h: lane 1, 1 h; lane 2, 2 h; lane 3, 3 h; lane 4, 4 h; lane 5, 5 h; lane 6, 6 h; lane 7, 12 h; lane 8, 18 h; lane 9, 24 h. Expression of JDV CA and CA/TM was detectable at all periods up to and including 24 h post-induction with no significant protein degradation. Fused JDV CAΔTM protein was detectable at 1, 2 and 3 h post-induction but started to degrade 2 h post-induction and was completely degraded 4 h post-induction.

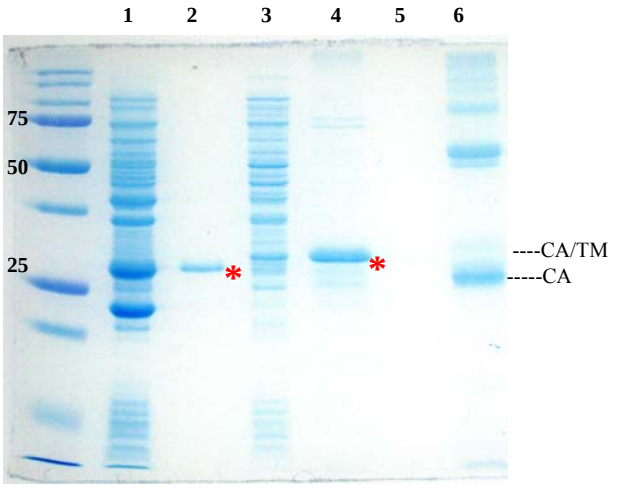


Figure 3.3. An SDS-PAGE gel showing the expression level and final purity of the JDV recombinant CA and CA/TM proteins. Lanes 1 and 3, total bacterial lysates expressing JDV CA and JDV CA/TM, respectively; lanes 2 and 4, Ni-NTA affinity purified JDV CA and JDV CA/TM, respectively; lane 5, empty and lane 6, JDV whole virus antigen preparation. Molecular weight markers are shown on the left hand side, and proteins of interest on the right hand side. Lanes 1 and 3 show the expression levels of the protein relative to whole bacterial lysate and lanes 2 and 4 show the relative purity of the final recombinant proteins.

Whole virus antigen Western immunoblots

Whole-virus Western immunoblots were conducted on 200 field serum samples as a reference immunoassay for the detection of antibody to bovine lentiviruses. The seroprevalence of JDV in the 200 cattle was 11.5% (Figure 3.4b). Of the 23 positive sera only one had a response to TM, and this sample also had a weak reaction to CA (Figure 3.4a).

a b

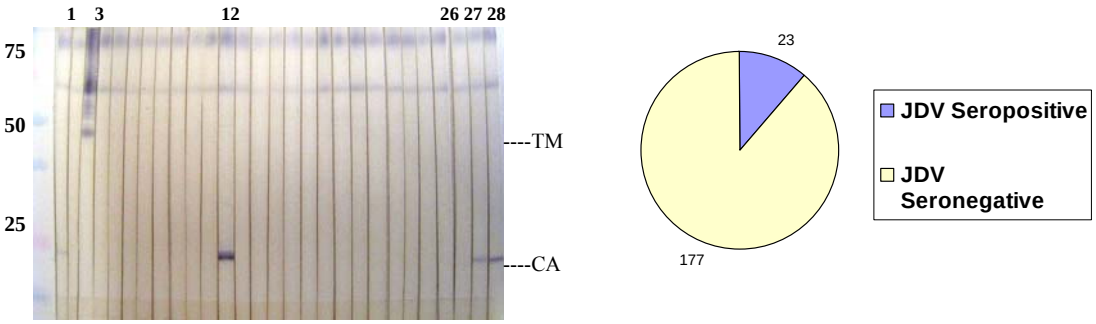


Figure 3.4. (a) An example of the whole virus Western immunoblotting of field sera, with selected lanes shown on top, the size of the selected molecular weight markers on the LHS, and proteins of interest on the RHS. All field sera shown on this Western immunoblot gave a negative reaction except for one serum with a weak CA reaction (lane 1), one reacting with TM (lane 3) and one with a strong CA reaction (lane 12). The reference sera included a negative serum (lane 26), a reference positive serum (lane 27) and serum from a hyper immunized animal (lane 28). (b) Graphical representation of the antibody status of the 200 sera that were examined.

Recombinant CA protein ELISA

Negative cattle sera (1:200 dilutions) were tested by ELISA using 200 ng/well of JDV CA to determine the cut-off values as the mean OD_{405} of the negative samples plus 3 SD of the mean. Positive sera from 10 experimentally infected and recovered cattle were also tested to determine specificity and sensitivity of the assay (Table 3.2 and Figure 3.5). All ELISA cut-offs were validated using TG ROC.

Whole-virus antigen Western immunoblotting was conducted on the 200 field serum samples and 23 of the sera (11.5%) reacted with the 26 kDa CA protein and one or more other JDV proteins (Figure 3.4). The CA ELISA detected 22 of the 23 Western immunoblot-positive sera with 1 false negative and 2 false positives giving a concordance of 98.5% with a kappa value of 0.9277. The specificity of the assay was 98.9% with a sensitivity of 95.8% using the previously described cut-off value of 0.499 at OD_{405} (Table 3.3 and Figure 3.6).

Table 3.2. Examples of descriptive indices for the JDV CA ELISA using reference negative and positive sera. In this series of assays, a cut-off of 0.499 was calculated from the mean OD_{405} + 3 SD of the mean of the reference negative samples, which provided a sensitivity and specificity of 100% for the reference positive serum samples.

Measurement	**Positive**	**Negative**
Number sera examined	10	30
Mean $OD_{405\ nm}$	1.79615	0.295662
Median $OD_{405\ nm}$	1.9985	0.2905
SD of mean	0.746806	0.067654
Minimum $OD_{405\ nm}$	0.7465	0.153
Maximum $OD_{405\ nm}$	2.593	0.4565

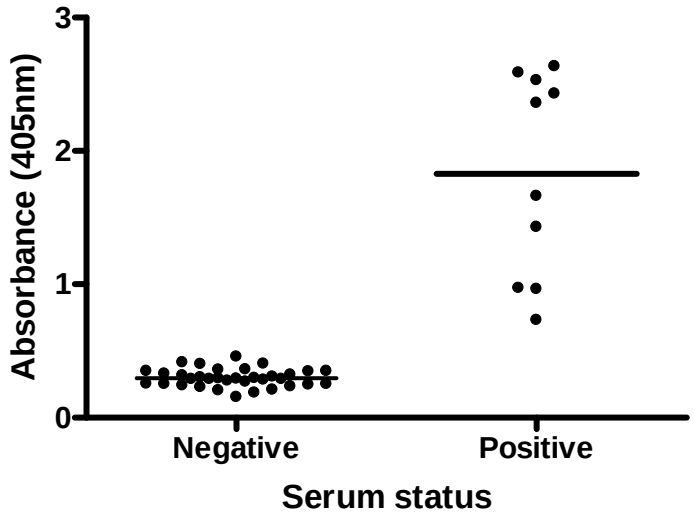


Figure 3.5. Scatter plot of ELISA OD_{405} with the recombinant JDV CA as a coating antigen using 30 reference-negative and 10 reference-positive sera (identified by whole virus Western immunoblotting). The central horizontal line represents the mean of the groups whilst each dot is representative of a single serum sample.

Table 3.3. Descriptive indices for the JDV CA ELISA using positive and negative field sera determined by whole virus Western immunoblotting. In this series of assays, a cut-off of 0.499 was calculated from the mean OD_{405} + 3 SD of the mean of the reference negative samples, which provided a sensitivity of 95.8% and specificity of 98.9%.

Measurement	**Positive**	**Negative**
Number of sera	23	177
Mean $OD_{405\ nm}$	1.406617	0.227673
Median $OD_{405\ nm}$	1.18815	0.2161
SD of mean	0.755021	0.102673
Minimum $OD_{405\ nm}$	0.5649	0.07315
Maximum OD_{405}	3.14895	0.63345

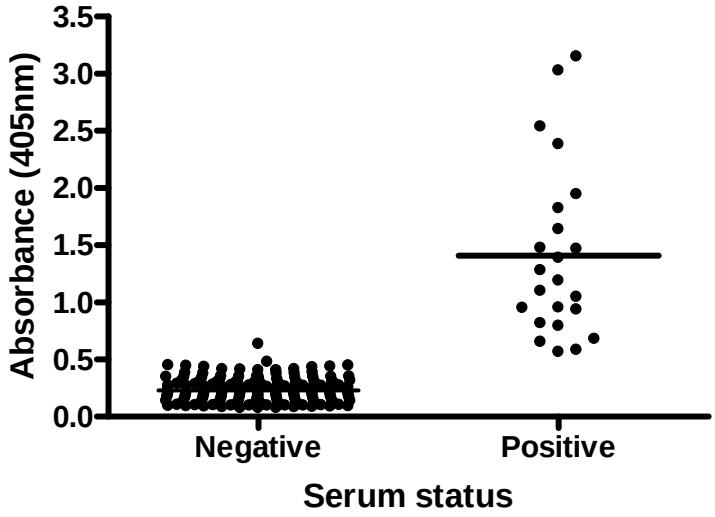


Figure 3.6. Scatter plot of OD_{405} of the JDV CA ELISA with 177 negative and 23 positive field sera (identified by whole virus Western immunoblotting). The central horizontal line represents the mean of the groups whilst each dot is representative of a single serum sample.

Recombinant CA/TM ELISA

The reference negative serum samples were tested by JDV CA/TM ELISA using 50 ng per well of protein to determine the cut-off value. The cut-off value with a 1:100 serum dilution was 0.557 (mean OD_{405} 0.28229 plus 3 SD [0.091588]) as shown in Table 3.4 and Figure 3.7. The reference positive sera all provided a positive result indicating a sensitivity and specificity of 100%.

Table 3.4. Descriptive indices for the results of the JDV CA/TM ELISA using reference negative and positive reference sera. In this assay, a cut-off of 0.557 was calculated using the mean + 3 SD of the mean of the reference negative samples, providing a sensitivity and specificity of 100% in the reference serum samples.

Measurement	**Positive**	**Negative**
Numbers sera examined	10	30
Mean OD_{405}	2.060695	0.28229
Median OD_{405}	2.13905	0.270525
SD of mean	0.941998	0.091588
Minimum OD_{405}	0.913	0.1213
Maximum OD_{405}	3.36315	0.47025

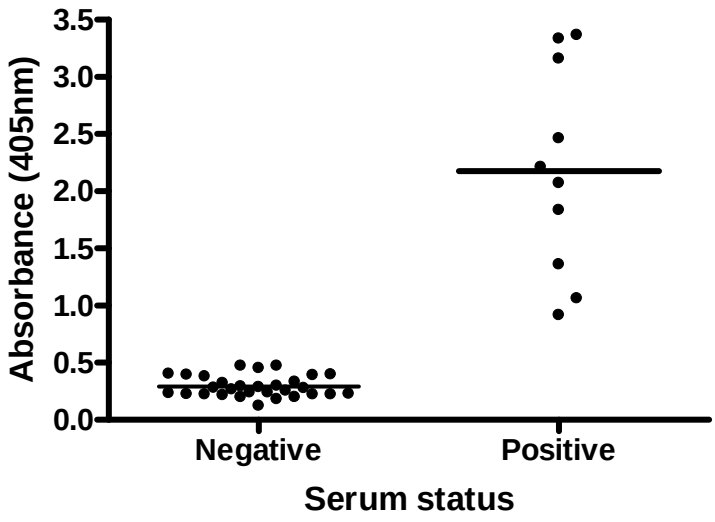


Figure 3.7. Scatter plot of OD_{405} of JDV CA/TM ELISA with 30 reference negative and 10 reference positive sera. The central horizontal line represents the mean of the groups whilst each dot is representative of a single serum sample.

The CA/TM ELISA detected 9 of the 23 whole virus Western immunoblot positive samples but failed to detect 14 of the other Western immunoblot positive samples (false negatives) and gave positive values with 4 other sera (false positives) with an overall concordance of 91% and a kappa value of 0.4547. The specificity of this assay was 97.8% with a sensitivity of 62.2% (Table 3.5 and Figure 3.8).

Table 3.5. Descriptive indices for the JDV CA/TM ELISA using field sera. A cut-off of 0.557 (mean OD_{405} + 3 SD of mean) provided a sensitivity of 62.2% and specificity of 97.8% when compared to the results obtained using whole virus Western immunoblots.

Measurement	**Positive**	**Negative**
Numbers sera examined	23	177
Mean $OD_{405\ nm}$	0.755376	0.267696
Median $OD_{405\ nm}$	0.4303	0.254075
SD of mean	0.719279	0.138756
Minimum $OD_{405\ nm}$	0.15995	0.06795
Maximum $OD_{405\ nm}$	2.4	0.7724

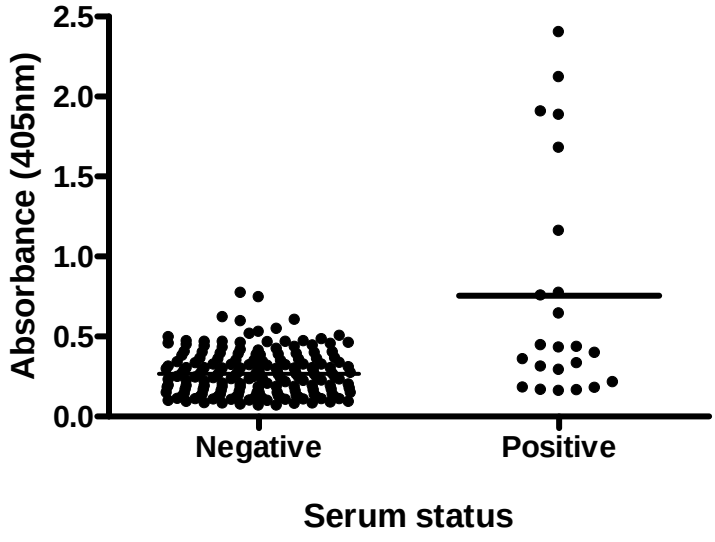


Figure 3.8. Scatter plot of OD_{405} for JDV CA/TM ELISA with 177 negative and 23 positive field sera (previously identified by a whole virus Western immunoblot). The central horizontal line represents the mean of the groups whilst each dot is representative of a single serum sample.

Western immunoblots with recombinant protein antigens

The JDV CA Western immunoblot had a specificity of 93.7% and a sensitivity of 88.5% with an overall concordance of 92.5% (kappa value 0.6851) when compared to the whole virus antigen Western immunoblot (Figure 3.9). The JDV CAΔTM Western immunoblot had a specificity of 95.7% and a sensitivity of 71.9% with an overall concordance of 91.5% when compared to the whole virus antigen Western immunoblot (kappa value 0.5743) (Figure 3.10).

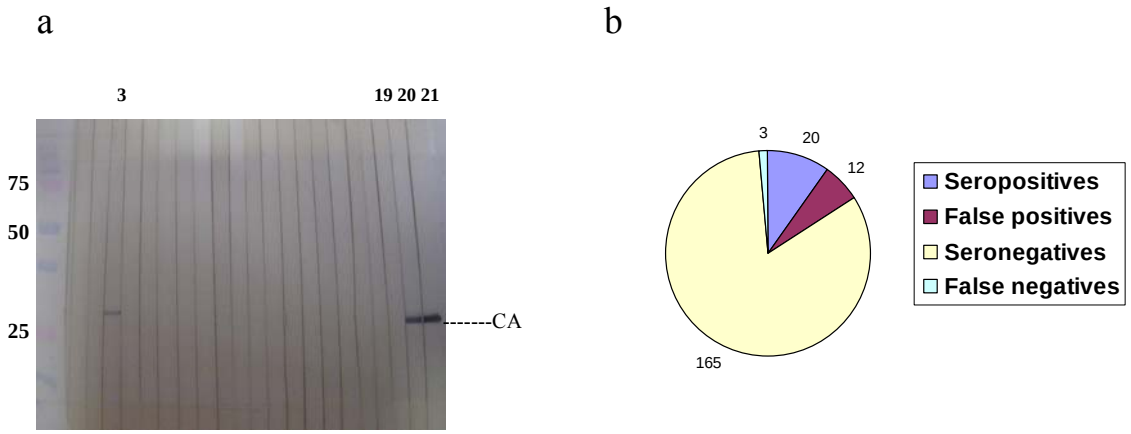


Figure 3.9. (a) Example of results of Western immunoblotting using the recombinant CA antigen, the size of the selected molecular weight markers on the LHS, and the protein of interest on the RHS. Lanes of interest are numbered (on top). All field sera (lanes 1-18) were negative except for the serum sample in lane 3. A reference negative serum is shown in lane 19, a reference positive serum in lane 20 and serum from a hyper-immunized animal in lane 21. (b) Graphical representation of the JDV antibody status of 200 field sera by JDV CA Western immunoblot by comparison to results obtained using a whole virus antigen Western immunoblot.

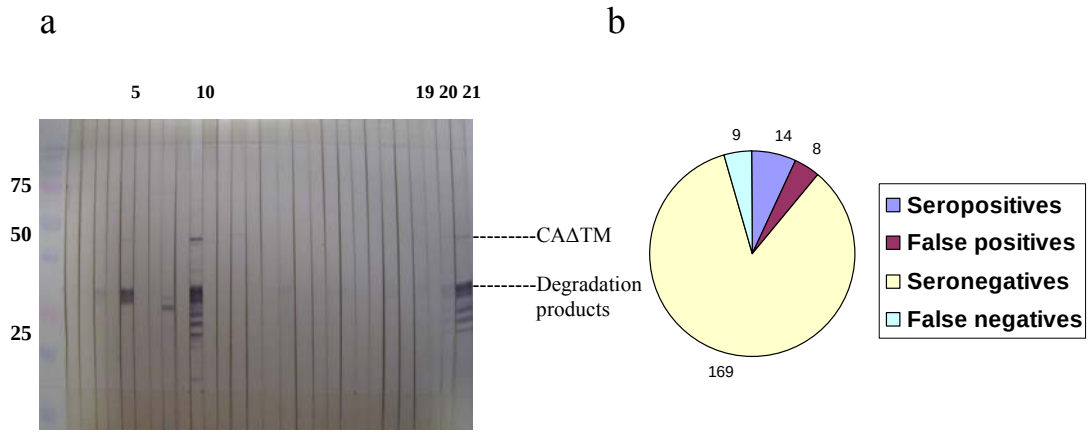


Figure 3.10. (a) Western immunoblot demonstrating typical results obtained using the CAΔTM recombinant protein antigen, the size of the molecular weight markers on the LHS, and the protein of interest on the RHS. In the Western immunoblot shown, all field sera were negative except one with a weak CA reaction (lane 5) and one with a strong CA reaction (lane 10). Also shown are a reference negative serum (lane 26), a reference positive serum (lane 27) and serum from a hyper-immunized animal (lane 28). (b) Graphical representation of the results obtained in 200 field sera by JDV CAΔTM immunoblotting by comparison to results obtained using a whole virus Western immunoblotting.

Discussion

Serological assays for JDV infection in cattle reported previously include an ELISA and an agar gel immunodiffusion test which made use of purified whole virus antigens (Hartaningsih et al., 1993; Hartaningsih et al., 1994) and an ELISA utilising recombinant protein antigens (Burkala et al., 1998) which detected antibodies against the individual CA and TM proteins. Preparation of a whole virus antigen for Western immunoblotting (Hartaningsih et al., 1995b) required ultracentrifugation facilities that are currently unavailable in Indonesia and limited supplies of this antigen have been utilized mainly for Western immunoblotting. For ELISA, a recombinant JDV CA-GST fusion protein antigen has been used (Burkala et al., 1998) but the GST tag attached to the CA protein is an antigenic *Schistosoma japonicum*-derived protein which gave a high rate of false positive results as cattle which have been infected with *Schistosoma japonicum* (endemic in Indonesia) react to the GST portion rather than the CA of the recombinant protein.

The current ELISA recombinant JDV CA protein with an *E. coli*-derived biotinylated tag was replaced with a full length JDV CA protein with a much smaller hexahistidine

tag. This 3 kDa hexahistidine residue was much smaller than the 13 kDa biotinylated *E. coli*-derived tag making it much less likely to be antigenic and result in false positive results. The hexahistidine tagged CA production was also less time intensive and expensive to produce than the biotinylated *E. coli*-derived CA protein with a greater final yield and purity. For comparison of various serological assays, generally a "gold standard" assay is used against which estimations of sensitivity and specificity have to be applied (Enoe et al., 2000; Greiner et al., 2000). The selection of a gold standard assay for JDV was problematic but as greatest reliance has in Indonesia been placed on the use of a whole virus Western immunoblot to confirm whole virus ELISA results, and more recently to confirm recombinant protein antigen ELISA results, this was used as the reference with which to compare the recombinant protein ELISA and Western immunoblots that were developed in this current study. This assay was unlikely to detect all JDV antibody-positive sera for several reasons: generally whole virus serological assays have good specificity but poor sensitivity (Brinkhof and van Maanen, 2007); there may be genetically and antigenically different strains of JDV in Indonesia, which would lead to false negatives, although JDV isolates from Bali appear to be remarkably genetically stable and this is unlikely to be a problem (Desport et al., 2007); studies by (Hartaningsih et al., 1994; Putra and Sulistyana, 1995) have shown that the antibody response in some cattle fluctuates from positive to negative which suggests the levels of circulating antibodies are close to the limit of detection of the assay.

In these current studies, recombinant CA and TM proteins were selected as targets for alternate antigens. A previous study showed that the CA and TM proteins were recognized for up to 21 weeks post-infection with the immunodominant response being to the CA protein (Hartaningsih, 1993). A further study demonstrated that the recombinant CA and TM proteins of JDV provided greater sensitivity for detection of JDV specific antibody than did the nucleocapsid, matrix and truncated surface envelope proteins (Lewis, 2004). These results are similar to what has been found with CAEV, MVV, FIV, BIV and EIAV for which the CA and TM antigens are most commonly selected as antigens for serological diagnosis (Calzolari et al., 1995; de Andres et al., 2005; Kwang et al., 1993; Saman et al., 1999). In JDV infections, an antibody response to the CA is generally the first detectable response and therefore JDV p26 is an essential component of a diagnostic assay for infections with this virus.

However, variable Gag responses have been reported after experimental infection with BIV and JDV. BIV CA antibodies were detectable from 2 weeks until at least 2.5 years post-infection (Whetstone et al., 1990a) and in another study (Isaacson et al., 1995a) Gag responses were found to have declined by 40 weeks post-infection and remained low or undetectable until 190 weeks post-infection. Serological detection of the genetically related small ruminant lentiviruses (SRLV) was improved by using the combination of *Maedi visna virus* (MVV) CA and a TM peptide as coating antigens in an ELISA (Celer and Celer, 2001). This has been further improved by expressing the entire CA and TM in a single fusion protein for serological detection of SRLV, FIV and EIAV infections (Rosati et al., 2004).

Generally there was a good concordance amongst all the assays with the strongly positive cattle but the CA/TM ELISA and the CAΔTM Western immunoblot failed to detect the borderline positives that were recognized by the CA ELISA and whole virus Western immunoblot (results not shown). The CA antigen ELISA showed an excellent agreement beyond chance (98.5% concordance with a kappa value of 0.9277) with the whole virus Western immunoblot in both the experimentally infected cattle (n=10) and the field serum (n=200) which indicated that this antigen can reliably replace the whole virus Western immunoblot for detection of JDV-infected cattle. Surprisingly, there was very little difference between the sensitivity and the specificity of the experimentally infected and the field sera whereas most lentiviral diagnostic assays have a decreased sensitivity and specificity with field sera, but this may be due to the small sample size (n=200) in this current study as generally greater sample sizes have been used (Brinkhof and van Maanen, 2007; de Andres et al., 2005; Greiner et al., 2000; Mboloi et al., 1999). This high level of concordance will have to be further tested in a larger epidemiological and statistical study to validate the sensitivity and specificity of the test. The current study was a preliminary investigation only as only 30 negative and 10 positive sera were used to test and validate the cut-offs for the various ELISA assays whilst the OIE recommends a minimum of 1000 negative sera and 300 positive sera (OIE Manual of Standards for Diagnostic Tests and Vaccines,(2000). Further investigation must also be undertaken on sera from fluctuating borderline antibody-positive cattle to determine if the assay fails to continuously detect antibody-positive cattle as was observed when the whole virus antigen was used (Putra & Sulistyana, 1995).

Western immunoblots with the JDV CA provided a specificity of 93.7% and a sensitivity of 88.5% with a good agreement beyond chance with a concordance of 92.5% with a kappa value of 0.6851 to the whole virus Western immunoblot. This specificity and sensitivity is within the normal ranges for other serological diagnostic assays for lentiviruses and is typical of other studies with CAEV/MVV and FIV where the CA Western immunoblot has identified more seropositive animals than ELISA (Calzolari et al., 1995; de Andres et al., 2005; Zheng et al., 2000). This could be due to higher sensitivity of the Western immunoblot when compared to indirect CA ELISA and whole virus Western immunoblotting but this is unlikely as there were 3 false negative sera which were detected by both whole virus Western immunoblots and the indirect CA ELISA. There may be some interfering factors present in certain cattle such as endogenous cross-reactive antibodies, maternal antibodies or reactive pathogens (Greiner and Gardner, 2000). Due to the time consuming nature of the assay and the lower correlation with the whole virus Western immunoblot it is recommended that this assay should only be used to confirm borderline ELISA results.

Attempts were made to incorporate the TM protein into the assays by producing a single fused CA and TM polyprotein antigen because earlier studies have shown that all experimentally infected cattle develop a TM response after infection (Ditcham, 2007). The fused CA/TM antigen ELISA provided only a low sensitivity when used to detect antibody in field sera, less than obtained with the whole virus Western immunoblot and recombinant CA ELISA (Greiner and Gardner, 2000). There was borderline poor to fair agreement beyond chance with a kappa value of 0.4547 between the CA/TM ELISA and the whole virus Western immunoblot. This assay also had the lowest concordance of 91% and the CA/TM ELISA failed to detect most borderline positives making it unsuitable for use in Jembrana disease surveillance. A possible reason for the loss of sensitivity may be due to a truncation of a portion of the carboxy terminus of the CA, which may contain previously unrecognized epitopes, or to alterations in the folding of the protein due to the presence of the TM peptide sequence. Further studies are needed to confirm if there is an important diagnostic epitope in this region. Other studies in small ruminant lentiviruses testing recombinant CA/TM fusion peptides have generally reported an increase in sensitivity with the fusion protein with values ranging from 64-100% sensitivity so the low sensitivity

was unexpected (de Andres et al., 2005; Saman et al., 1999). A full length CA/TM peptide construct should be developed and tested to see if this improves the sensitivity of the assay in further studies.

The TM portion of the CAΔTM construct was truncated to increase the expression and stability of the TM protein. Unfortunately, even though the CAΔTM protein expression levels were higher than previous constructs the stability of the protein was still poor with protein degradation occurring within 1 h post-induction. The JDV CAΔTM Western immunoblot had a low concordance with the whole virus Western immunoblot of 91.5% with a fair agreement beyond chance of 0.5743. This low concordance and agreement beyond chance was associated with all of the constructs that contained any portion of the TM protein and may be associated with the binding properties of this protein altering the conformation of the final protein. This is supported by the observation that when the *tm* gene was added to the CA construct there was a dramatic reduction in protein expression, stability and purity. It is important to note that one study of MVV found a similar lack of sensitivity of the combined CA/TM fusion protein with sensitivity dropping from 88% using the indirect whole virus ELISA to 64% with the indirect fusion protein ELISA (DeMartini et al., 1999). Due to stability and expression problems with the protein it provided the least reliable of the all the diagnostic ELISA reagents.

In conclusion, the indirect CA ELISA had the highest concordance to the whole virus Western immunoblot and appeared to be the most sensitive and specific of all the assays tested whilst the CA Western immunoblot may be useful for determining the status of borderline sera. Neither of the fusion proteins increased sensitivity or specificity and were difficult to express, stabilize and purify making them less desirable as diagnostic reagents. This assay is the first recombinant protein assay with a high concordance to the whole virus assays and reduced dramatically the incidence of false positives due to cross reactive tag proteins. It appeared from this study that the JDV indirect CA ELISA could be used to replace the whole virus Western immunoblot for the detection of antibodies to JDV.

Chapter 4. Production of a novel recombinant JDV Capsid and Tat polyprotein as a potential vaccine for Jembrana disease

Summary

Methods were determined for the small scale production of a GST-tagged recombinant polyprotein containing both JDV CA and Tat proteins, with potential for use as a vaccine for the control of Jembrana disease. A series of quality control procedures including sterility testing, methods for the quantification of protein and methods for the detection of nucleic acid and endotoxin contamination, were also developed for the expressed and purified protein. In small scale experiments, the CA/Tat-GST polyprotein was produced with yields of up to 1.2 g/L of 95% pure recombinant protein. Endotoxin contamination was detected at a level of 4 EU/mg of recombinant polyprotein protein. Nucleic acid contamination was also detected but was completely removed by treatment with 75 U/mL of benzonase at room temperature for 45 min.

Introduction

There are several advantages to the use of recombinant proteins for vaccines. They are generally easy to produce at a relatively low cost and they have been successfully used for many virus induced diseases (McElrath et al., 2000; Pauza et al., 2000; Uhl et al., 2002). Recombinant GST-tagged proteins JDV capsid (CA), matrix (MA), surface unit (SU), transmembrane (TM) and Tat expressed in *Escherichia coli* have been tested as potential vaccines against Jembrana disease in Indonesia, where it has been possible to immunize susceptible cattle and then challenge them with live virus under controlled conditions. When a subunit vaccine consisting of a combination of recombinant JDV CA and Tat in incomplete Freund's adjuvant (IFA) was administered 3 times, it was found to decrease the severity of the febrile response and leucopenia that typically occur during Jembrana disease and to also cause a significant reduction in virus load during the early phase of disease (Ditcham, 2007).

Concurrent with the high virus load in plasma during the acute disease process, the CA protein, the major structural protein of the virus, can be detected at high levels in the serum of infected cattle (Stewart et al., 2005). The CA protein is also the immunodominant protein in the tissue-derived inactivated virus vaccine that has been shown to ameliorate the clinical signs of Jembrana disease in vaccinated cattle (Hartaningsih et al., 2001). While it is difficult to detect Tat in tissues of cattle during Jembrana disease (Setiyaningsih et al., 2008) the tat protein is extremely important in replication of bovine lentiviruses. Tat has been shown to transactivate the viral LTR needed for transcription of viral genes (Barboric et al., 2000; Chen et al., 1999; Jeang et al., 1999), and regulates many cellular genes such as cytokines, oncogenes and adhesion molecules (Kashanchi et al., 2000; Marcello et al., 2001), acts as a potent extra-cellular chemo-attractant for virally susceptible cells, and induces apoptosis in bystander cells (Albini et al., 1998; Bartz and Emerman, 1999; Giacca, 2005; Marchio et al., 2005; Yang et al., 2003). JDV Tat is the most potent of all the characterized lentiviral Tat proteins and has been shown to transactivate other lentiviral LTR regions using both the primate cellular cyclin T1 protein-dependant binding to the transactivation region and directly to the bovine transactivation region adopting the ß-hairpin conformation needed for transactivation (Chen et al., 1999; Deng et al., 2006; Xie et al., 2004; Xie et al., 2003). The diverse but essential biological functions of Tat and the low natural antigenicity make Tat an ideal vaccine candidate, and there are currently many HIV vaccine trials and publications describing the use of Tat as a potential vaccine (Fulcher and Jans, 2003; Pauza et al., 2000; Theisen et al., 2006). While Tat is potentially encoded by 2 ORF's, *tat*1 and *tat*2, in JDV there is an in-frame stop codon prior to the splice donor on exon 1 suggesting that only *tat*1 is translated during the acute phase of infection (Setiyaningsih et al., 2008). The Tat1 protein of JDV is 97 amino acids with a predicted molecular weight of 10.7 kDa and was selected as the potential vaccine candidate, rather than Tat2, as it is known to be translated during peak viraemia and is sufficient on its own to generate a functional Tat (Chen et al., 1999).

To further investigate the value of combinations of recombinant CA and Tat proteins for use as vaccines for the control of Jembrana disease in Indonesia, methods to produce the recombinant proteins at the lowest cost possible were required. Only a low cost vaccine could be expected to have significant acceptance and use in

Indonesia. It was considered that production of 2 separate vaccine components would be likely to make production costs too high and an attempt was therefore made to express both the proteins as a single polyprotein so the vaccine could be produced in a single operation, thereby reducing production costs and increasing the likelihood of successful implementation of the vaccine. Although there appears to be no precedent for the use of an *E. coli*-expressed recombinant polyprotein vaccine, there are numerous examples of DNA lentivirus vaccines expressing multiple proteins (Burgers et al., 2006; Kong et al., 2003) and this is the first trial with an *E. coli* expressed recombinant polyprotein containing 2 fused viral proteins as a vaccine. Rigorous quality control procedures would be required if the polyprotein were to be produced commercially for use as a vaccine, involving tests for sterility, methods of quantification of the proteins, stability of the proteins and the detection of nucleic acid and endotoxin contamination (Aucouturier et al., 2001; Fanales-Belasio et al., 2002; Hansson et al., 2000; Tomasulo et al., 1977).

The investigation into the expression of a single fused CA and Tat polyprotein, and requisite quality control procedures, are reported in this Chapter. Analysis of the immunological responses of cattle to this polyprotein is reported separately in Chapter 5.

Materials and methods

Serum samples

Serum from a well-characterized long-term experimentally-infected heifer (A4) was used as the positive control for all ELISA and Western immunoblots.

A second positive control was taken from a hyper-immunized heifer (A577) and used in Western immunoblotting to try and detect responses against all viral proteins. This animal was vaccinated weekly for a 5-week period with inactivated virus as described by Hartaningsih *et al.* (2001).

Serum from a heifer (CB76) vaccinated 3 times with the tissue-derived inactivated whole virus in a mineral oil adjuvant as described by Hartaningsih *et al*. (1995) was also used to confirm the antigenicity of the fused protein.

Serum from an animal (CB92) immunized 3 times with 2 mg of recombinant CA-GST and 2 mg of recombinant Tat-GST in incomplete Freund's adjuvant (IFA) was also used to confirm the antigenicity of the fused protein (Ditcham, 2007).

Expression and purification of individual recombinant GST-tagged JDV CA and Tat proteins

Recombinant CA and Tat proteins were expressed as single proteins from JDV_{Tab87} genes previously ligated into pGEX6-P-1 (Pharmacia) by Burkala et al. (1998) and Setiyaningsih (2006) and the constructs were designated JDV CA-GST and JDV Tat-GST, respectively. Proteins expressed in this bacterial system are N-terminally fused to glutathione-S-transferase (GST), to aid in purification and identification. The constructs contained the full-length JDV *capsid* gene (Chadwick et al., 1995b) and exon1 of the JDV *tat1* gene (nt 5010-5299) (Setiyaningsih et al., 2008).

Recombinant JDV CA-GST and JDV Tat-GST proteins were expressed in BL21 *E. coli*. For expression of the CA-GST protein, bacteria were grown to an OD_{600} of 1.0 in 2YT broth plus 1 mM ampicillin and expression induced by the addition of 0.1 mM IPTG (final concentration) for 6 h before the cells were centrifuged and then lysed to release the recombinant protein. For expression of the Tat-GST protein, bacteria were grown to an OD_{600} of 1.5 in 2YT broth plus 1 mM ampicillin and induced by the addition of 0.1 mM IPTG (final concentration) for 6 h before centrifugation and cell lysis. The proteins from the lysed pellets were in an insoluble form, and were initially purified by 4 wash steps (15 min each on a rotary washer at 4ºC) with a 1% Triton X-100 wash buffer (Tris-HCl 10 mM, EDTA 5 mM and 1% [w/v] Triton X-100) and then the insoluble protein was solubilised with a 2 M urea/Tris based solution (urea 2 M, Tris 2 M, DTT 20 mM and PMSF 1 mM). The purified proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue to determine yield and purity (using procedures described below). Western immunoblots were also performed to confirm the reactivity of the recombinant proteins with sera from cattle naturally infected with JDV, cattle vaccinated with a whole virus vaccine (Hartaningsih, 1993) and cattle immunized with recombinant CA and Tat proteins.

Production of a single **c**onstruct (JDV CA/Tat) for expression of GST-tagged CA and Tat polyprotein

A pTrcHisCA/Tat construct was initially produced for expression of a histidine-tagged JDV CA/Tat polyprotein. Briefly, the *capsid* gene was amplified by standard PCR from JDV_{Tab87} plasmid 139# (Chadwick, 1995) with the primers shown in Table 4.1. The PCR product was then digested with BamH1 and then cleaned using a PCR clean-up kit (QIAGEN). The pTrcHis-Tat plasmid (Setiyaningsih et al., 2008) was also digested with BamH1 and the purified PCR product was ligated into the digested pTrcHisTat vector by a standard ligation reaction. The re-ligated plasmid was then transformed into Top10 *E. coli* cells using standard transformation conditions. The positive clones were identified by directional PCR and then cultured and glycerol stocks prepared. The clones were grown overnight in 2YT plus ampicillin (1 mM) and plasmid was purified using a Plasmid Miniprep Kit (QIAGEN). This purified plasmid was transformed into BL21 *E. coli* using standard transformation conditions and directional PCR with the primers shown in Table 4.1 was used to identify the clones containing the recombinant plasmid.

The expression levels of pTrcHisCA/Tat were low and to improve expression the insert was then ligated into a pGEX6-P-1 plasmid and this construct transformed into Top10, BL21 and JM109 *E. coli* for expression of a GST-tagged CA/Tat polyprotein. Briefly, the insert was amplified with JCaBamF and JTatBamR primers (Table 4.1) using Pfu DNA polymerase, from the pTrcHisCA/Tat construct. This PCR product was then digested with Sma1 and cleaned with a PCR clean up kit (QIAGEN). The pGEX6-P-1 plasmid was digested with Sma1 using a standard digestion reaction and the purified JDV *capsid/tat*1 fragment ligated in using a standard ligation reaction. The purified plasmid was then transformed into Top10, BL21 and JM109 *E. coli* and positive colonies were selected by directional PCR. Correct protein expression was determined using the methods described in Chapter 3 and clones providing high level of expression of recombinant protein were chosen and glycerol stocks of them were prepared stored at -80˚C until required. The plasmid was sequenced to confirm that both proteins would be expressed in-frame.

Table 4.1. Primer sequences used for the amplification, sequencing and development of recombinant plasmid constructs.

Primer	Sequence (5′ → 3′)	Genome	Nucleotide position in JDV_{Tab87}
jCaBamF	GACCGGatccCCACAACTTAGAAAGAACTTC	JDV	604-624
jCaBamR	GGatccCAAGAATTGCATCTTCTGT	JDV	1222-1204
jTatBamR	GACCGGatccGCGCAGTTAGGTGCCC	JDV	5299-5283
jCa604F	CCCACAACTTAGAAAGAAC	JDV	604-622
jCa1030R	CGGTCATGTTATCCTTGTGC	JDV	1030-1011
pGexF	CCGGGAGCTGCATGTGTCAGAGG	pGEX-6P	869-891
pGexR	GGGCTGGCAAGCCACGTTTGGTG	pGEX-6P	1056-1034

Expression of GST-tagged fused JDV CA/Tat polyprotein

BL21 *E. coli* stably transformed with the JDV CA/Tat construct were grown to an OD_{600} of 1.5 in 2YT medium plus 1 mM ampicillin and expression of the JDV CA/Tat protein was induced by the addition of 0.1 mM IPTG (final concentration) for 12 h. The cells were then lysed by sonication (8 cycles of 30 seconds sonication and 30 seconds rest) and centrifuged (10,000 g for 15 min). The polyprotein was in an insoluble form and the centrifuged pellet was then further purified by 6 wash steps (1 h each on a rotary washer at 4ºC), the first 2 washes with a 2% Triton X-100 wash buffer (Tris-HCl 10 mM, EDTA 5 mM and 2% [w/v] Triton X-100) followed by centrifugation (10,000 g x 15 min), then 2 washes with wash buffer containing 1% Triton X-100 and a further 2 washes with a wash buffer containing 0.5% Triton X-100. The final pellet was solubilised with a 2 M urea/Tris solution overnight (urea 2 M, Tris 2 M, DTT 20 mM and PMSF 1 mM). A 10 µL sample of the purified and solubilised protein preparation was subjected to SDS-PAGE and the polyprotein identified by Coomassie Brilliant Blue staining to enable the yield and purity of the protein to be determined. Western immunoblots were also performed to confirm the reactivity of the recombinant polyprotein with sera from cattle experimentally infected with JDV, cattle vaccinated with a whole virus vaccine and cattle immunized with recombinant CA and Tat proteins (Ditcham, 2007; Hartaningsih, 1993).

Several methods to lyse the bacterial cells and release the insoluble protein were compared to sonication as described above, including freeze-thawing (5 cycles of -186˚C to +42˚C), the addition of various concentrations of Triton X-100 and the use of Bugbuster® (Novagen) a proprietary preparation containing a mix of detergents and benzonase for which full details were unavailable. Triton X-100 at 0.5, 1.0, 2.0 and 5.0% concentrations in wash buffer (Tris-HCl 10 mM, EDTA 5 mM and 0.5-5% [w/v] Triton X-100) was added to cell pellets prepared from 100 mL of the same culture, for 30 min on a vertical mixing rotor at 4ºC. After treatment, all samples were washed as described above. A sample of each was then plated onto 2YT plates and grown overnight at 37 ºC to detect viable bacteria. A second sample was stained with 0.4% trypan blue (Sigma Aldridge) to detect viable cells.

Production of non-GST tagged proteins for qualitative analysis of the serological response to GST-tagged proteins antigens

Recombinant proteins with fusion tags other than GST were also prepared to analyse the antibody responses specific to the CA and Tat components of the vaccine.

A recombinant biotinylated JDV Tat1 was expressed using a Pinpoint (Promega) expression system, with a recombinant plasmid construct jTat1 (Figure 2.2) provided by Dr Moira Desport (this laboratory) that contained *tat*1 (nt positions 5,010-5,303) of the JDV_{Tab87} genome (Chadwick et al., 1995a). This insert was expected to encode a protein of 92 amino acids. Optimum expression of Tat was achieved by inoculation of a 1:50 volume of overnight culture of BL21 *E. coli* in 2YT broth plus 1 mM ampicillin, then allowing the *E. coli* to replicate to an OD_{600} of 0.6 before induction of expression by the addition of IPTG (0.1 mM final concentration) for 4 h. The majority of the protein was expressed in an insoluble form in inclusion bodies (Ditcham, 2007). To purify the insoluble protein in inclusion bodies, the bacterial cells were resuspended in cell lysis buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl and 5% [v/v] glycerol) and lysed by sonication (as described above). The suspension was then centrifuged (10,000 g for 15 min) and the lysed cell pellet containing the inclusion bodies was resuspended in 10 mL of PBS (adjusted to pH 7 with HCl) and agitated overnight at 4°C on an orbital mixer. The cell pellet was collected by centrifugation (10,000 g for 15 min at 4°C). The pellet was washed 4 times (10-30 min per wash in PBS [pH 7]) at 4°C followed by centrifugation (10,000 g for 15 min). Nine mL of solubilisation buffer (urea 2 M, Tris 2 M, DTT 20 mM, PMSF 1 mM)

was added to the washed pellet and resuspended by agitation on an orbital mixer at 4°C for 2 h. The solubilised pellet was added slowly to 390 mL of Refolding Buffer (Tris pH 8 100 mM, 350 mg CHAPS and 0.53 g DTT made up to 350 mL with deionized water) and the refolded protein was concentrated in an ultrafiltration cell (Amicon) until a volume of 20 mL was obtained. The final protein was filtered through a 0.22 μM exclusion filter (Millipore) to remove aggregated proteins and then dispensed into 1 mL amounts and stored at -80°C.

Recombinant histidine-tagged JDV CA was produced from a pTrcHis C plasmid kindly supplied by Margaret Collins (Royal Veterinary College) containing the entire JDV *capsid* sequence (Barboni et al., 2001) and purified as previously described (Chapter 3). Western immunoblots with experimentally infected and vaccinated animal sera were also performed to confirm antigenicity of the protein.

Quality control assays of the GST-tagged recombinant protein preparations

Determination of sterility of recombinant proteins

All purified protein preparations were plated in triplicate on 2YT agar and 2YT agar plus 1 mM ampicillin agar and incubated at 37ºC for 2 days. Protein preparations were also inoculated into triplicate 2YT and 2YT plus 1 mM ampicillin broths and incubated overnight at 37ºC at 225 rpm on a bench top shaker, and then forwarded to a commercial diagnostic laboratory for bacterial culture.

Quantification and stability of recombinant proteins

Densitometry was used to quantify the concentration of protein in the protein preparations, as described in Chapter 3. To determine recombinant protein stability all proteins were diluted to 2 mg/mL with or without the addition of 5% glycerol to help stabilize the protein and prevent degradation. These proteins were left for 1 year at 4°C and quantified by densitometry to determine percentage degradation and percentage stability.

Determination of purity of recombinant proteins

The proteins of interest were visualized by SDS-PAGE using a resolving gel (12.5% [w/v]) and a stacking gel (4% [w/v]) in a mini-gel (BioRad) system containing 0.1% SDS [w/v] as described by (Laemmli, 1970). Molecular weight markers (Precision

Plus, 100 kDa; BioRad), 5 µL/lane, were added to a marker lane and the protein of interest (diluted 1:1 in 2X loading buffer) was loaded into a single large well of a Mini Protean II cell (BioRad). Electrophoresis was for 54 min at 200 V with a running buffer consisting of 25 mM Tris, 192 mM glycine and 1% [w/v] SDS. Gels were removed and either stained with Coomassie Brilliant Blue or Western blotted onto nitrocellulose membranes. For Coomassie Brilliant Blue staining, gels were added to 50 mL of Coomassie Brilliant Blue stain (45% [v/v] methanol, 45% [v/v] distilled water, 10% [v/v] glacial acetic acid and 0.05% [v/v] Coomassie Brilliant Blue) and left overnight at room temperature on a horizontal rocker. The excess stain was removed using a de-stain solution (40% [v/v] methanol, 50% [v/v] distilled water and 10% [v/v] glacial acetic acid) which was replaced at least twice until the gel was fully de-stained. A gel drying solution (20% [v/v] ethanol, 3% [v/v] glycerol and 77% [v/v] distilled water) was then added to the gel and left for 30 min. The de-stained gels were then dried between cellulose sheets and stored. The identity of the proteins was confirmed by Western immunoblotting as described in Chapter 3.

Detection of nucleic acid contamination in recombinant proteins

The solubilised recombinant proteins were tested for DNA and RNA contamination using a NanoDrop® ND-1000 kit (Biolab). A range of dilutions of the protein from 1:2 to 1:32,768 were tested and a mean nucleic acid concentration obtained. To determine if any of the DNA/RNA from the bacterial cells was of plasmid origin, PCR assays were performed on the dilutions using pTrcHisF and pTrcHisR primers (Table 4.1).

Benzonase treatment of recombinant proteins to degrade contaminating nucleic acid

Benzonase is used in large scale production of vaccines to degrade contaminating RNA and DNA. The benzonase protocol (Novagen) recommended a working concentration of 50 enzyme units/mL for 10 min at room temperature to degrade all nucleic acid. This proved insufficient for the plasmid nucleic acid and extra benzonase was added to the lysis buffer to determine the optimal concentration and time for complete nucleic acid degradation. Incubation times of 15, 30 and 45 min were tested with concentrations of benzonase of 50, 55, 60, 75, 100, 150 and 500 U/mL of solubilised CA/Tat-GST protein.

Assay of recombinant proteins for endotoxin

The presence of endotoxin in the JDV recombinant proteins was detected using an E-Toxate® (Limulus Amoebocyte lysate) (LAL) assay (Sigma Aldrich) as recommended by the manufacturer. The assay was performed under stringent aseptic conditions with pyrogen-free glassware, plastic ware and reagents to avoid endotoxin contamination. Standard quantities of endotoxin were used to create a standard curve from which results obtained with serially diluted protein samples were compared (Tomasulo et al., 1977). All controls were as described by the manufacturer and the standards provided results within the range expected.

Assay of recombinant proteins for cytotoxicity

Cytotoxicity of the CA/Tat-GST polyprotein was detected using the Celltiter96®Aqueous One Solution Cell Proliferation Assay (Promega) which determined changes in the number of viable cells exposed to the protein. Two standard curves were produced as described by the manufacturer, one for African green monkey kidney (VERO) cells and a second for primary foetal bovine lung (FBL) cells. To each well of a 96-well microtitre cell culture tray, 50,000 cells were added and left for 4 h at 37ºC in the presence of differing concentrations of JDV CA/Tat-GST, CA-GST and GST only (negative control). All samples were tested in quadruplicate and a mean was obtained which was then plotted on a graph with a downward trend indicating cytotoxicity and an upward trend indicating a proliferative response. The absorbances were then plotted on the standard curves of the VERO and FBL cells and the total number of cells/well was determined. The standard curve of the VERO cells had an R^2 value of 0.9572 and the equation used for determining the number of cells/well was OD at a wavelength of 490 nm (OD_{490}) =0.0043X+0.2952. The standard curve of the FBL cells gave an R^2 value of 0.9856 and the equation for determining the number of cells/well was A_{490} =0.003X+0.2452.

Results

Optimisation of JDV CA/Tat-GST polyprotein expression

A growth curve for the cultures inoculated with 3 different dilutions of overnight inoculum are shown in Figure 4.1 .Exponential growth slowed 4-6 h post-inoculation and started to plateau about 7 h post-inoculation, irrespective of the initial dilution of starter culture.

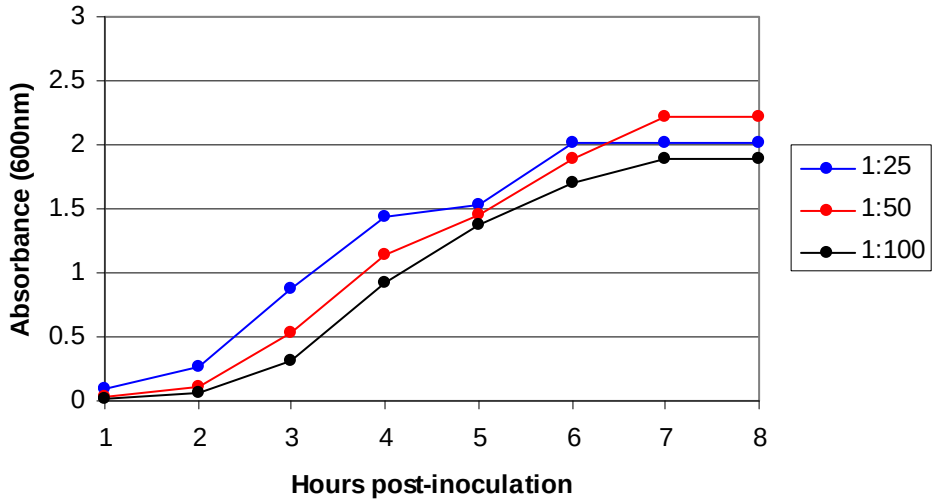


Figure 4.1. The effects of differing dilutions of inoculum on the growth curve of BL21 *E. coli* containing the CA/Tat-GST constructs grown in 2YT with 1 mM ampicillin. Three different dilutions of the inoculum were added and the growth dynamics of the bacteria containing the recombinant plasmid was determined.

The expression of the CA/Tat-GST polyprotein after induction with IPTG of cultures at an OD_{600} of 1 and 1.5 for periods from 1-24 h is shown in Figures 4.2 and 4.3. From the data, induction of expression of cultures at an OD_{600} of 1.5 for 12 h was chosen for further recombinant protein production.

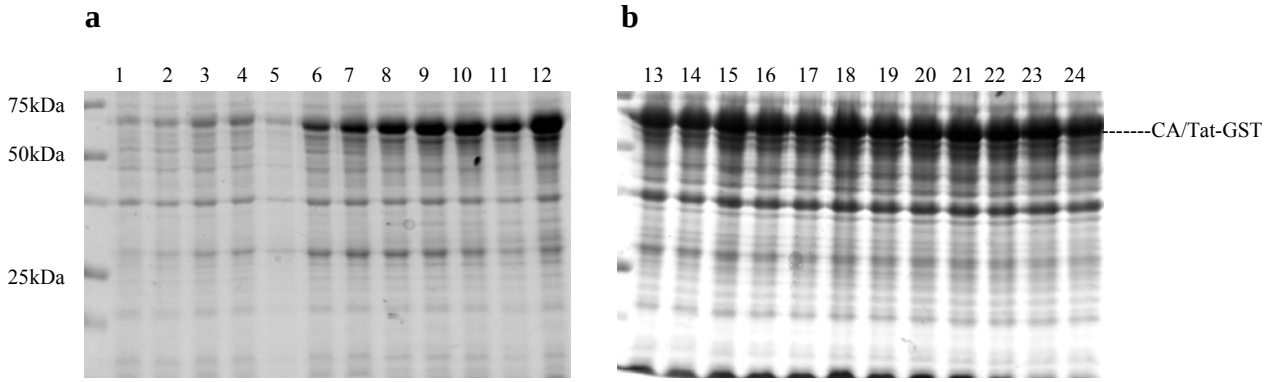


Figure 4.2. SDS PAGE gel showing the relative expression of the CA/Tat-GST polyprotein in BL21 *E. coli* grown in 2YT and induced with IPTG at an OD_{600} of 1.0. (a) Lanes 1-12, after expression for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 h respectively. (b) Lanes 13-24, after expression for 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 and 24 h, respectively.

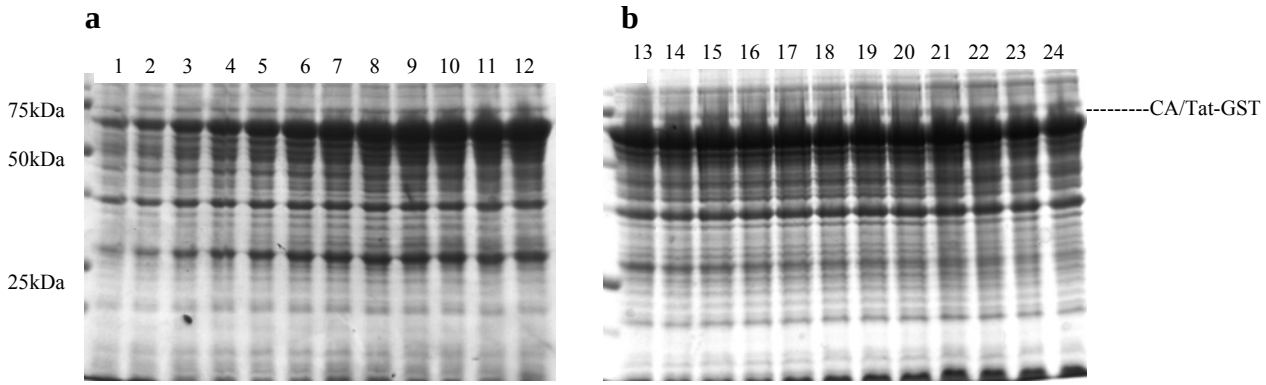


Figure 4.3. SDS PAGE gel showing the relative expression of the CA/Tat-GST polyprotein in BL21 *E. coli* grown in 2YT induced with IPTG at an OD_{600} of 1.5. (a) Lanes 1-12, after expression for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 h, respectively. (b) Lanes 13-24, after expression for 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 and 24 h, respectively.

Comparison of methods of chemical cell lysis

Ultrasonication resulted in total lysis of bacterial cells as indicated by the inability to detect viable cells by either culture or trypan blue staining. Similar results were obtained after freeze-thawing and when a combination of ultrasonication and freeze-thawing was used. BugBuster® resulted in complete lysis of the bacterial cells within the 30 min period recommended by the manufacturer. Triton X-100 at concentrations of ≥ 1% for 30 min provided complete cell lysis comparable to sonication,

freeze/thawing or commercial cell lysis solutions (Table 4.2). Suspensions treated with 0.5% Triton X-100 for 30 min still had detectable colonies on the 2YT agar plates (30 colonies/mL) indicating that concentrations of Triton X-100 less than 1% were insufficient to lyse all cells in a 30 min period. None of the treatments with Triton X-100 appeared to affect the final yield of protein, although BugBuster® did appear to reduce yield but full quantification of this was not attempted.

Table 4.2. Degree of lysis detected after treatment of BL21 *E .coli* expressing the recombinant JDV CA/Tat-GST protein with various concentrations of Triton X-100 for periods up to 30 min, compared to a commercially available chemical lysis method (BugBuster®). As an index of cell lysis, the presence of viable cells was determined by trypan blue staining the cells and determining the percentage viable bacterial cells.

Duration of treatment (min)	Triton X-100 (%)				BugBuster® (Novagen)
	0.5	1	2	5	
5	No lysis	No lysis	No lysis	~30%	NT[a]
10	~10%	~15%	~30%	~95%	NT
20	~15%	~30%	~50%	Complete	NT
30	~25%	~40%	Complete	Complete	Complete

[a] NT denotes not tested

Purification and quantification of the recombinant protein

The JDV CA/Tat-GST polyprotein was not solubilised during the 6 washes with wash buffers containing Triton X-100 but was solubilised by the addition of urea, with ~95% purity as shown in Figure 4.4. The predicted yield of the polyprotein from 1 L of culture, deduced from the batch shown in Figure 4.4 was 676 mg (Table 4.3).

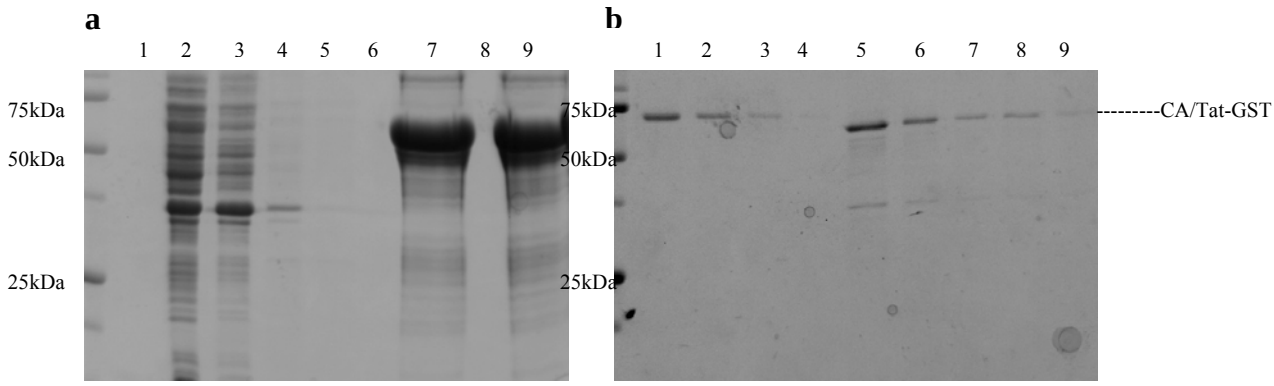


Figure 4.4. (**a**) Coomassie Brilliant Blue stained SDS-PAGE gel of the second, fourth and sixth wash fractions and the final purified protein. Lane 1, no product; lane 2, wash buffer from the second wash in wash buffer containing 2% Triton X-100; lane 3, wash buffer from the fourth wash in wash buffer containing 1% Triton X-100; lane 4, wash buffer from the sixth (final) wash in wash buffer containing 0.5% Triton X-100; lane 5, wash buffer consisting of PBS after 1 wash; lane 6, no product; lanes 7 and 9, CA/Tat-GST polyprotein after solubilisation in solubilisation buffer containing 2 M urea, lane 8, no product. The results demonstrate that the CA/Tat-GST polyprotein was not solubilised during washing steps with buffers containing 2% or less Triton X-100 (lanes 2, 3 and 4) or PBS (lane 5) but that most of the contaminating *E. coli* proteins were removed by washing (lanes 7 and 9). (**b**) An example of a Coomassie Brilliant Blue stained SDS-PAGE for quantification of the CA/Tat-GST polyprotein by densitometry, and for assessing the purity of the product. Lane 1, 1000 ng BSA; lane 2, 500 ng BSA; lane 3, 250 ng BSA, lane 4, 125 ng BSA, lane 5, 1 µL of CA/Tat-GST preparation; lane 6, 0.5 µL of CA/Tat-GST preparation; lane 7, 0.25 µL of CA/Tat-GST preparation; lane 8, 0.1 µL of CA/Tat-GST preparation; lane 9, 0.05 µL of CA/Tat-GST preparation.

Table 4.3. Examples of the final protein concentration, purity and expected yield obtained with various recombinant protein preparations produced in small scale cultures.

Protein	Concentration	Purity	Yield/L
Tat-GST	8,248 ng/μL	<85%	80 mg
CA-GST	6,456 ng/μL	<85%	65 mg
CA/Tat-GST	6493 ng/μL	>95%	676 mg

The production of a recombinant GST-tagged CA/Tat polyprotein instead of separate GST-tagged CA and Tat proteins would have resulted in an overall increase in the ratio of CA and Tat relative to the GST component of the polyprotein (Table 4.4).

Table 4.4. Comparison of the content of each component protein/mg in the recombinant protein preparations. A 4 mg quantity of JDV CA/Tat-GST polyprotein (the anticipated dose per vaccination) contained 1.6 mg of CA, 0.67 mg of Tat and 1.73 mg of GST, whilst 2 mg of the individual JDV CA-GST contained 0.96 mg of CA and 1.04 mg GST, and 2 mg of JDV Tat-GST contained 0.55 mg of Tat and 1.45 mg GST).

Protein	Content per mg of expressed protein		
	CA	Tat	GST
CA/Tat-GST polyprotein	0.4 mg	0.1675 mg	0.4325 mg
CA-GST	0.48 mg	-	0.52 mg
Tat-GST	-	0.275 mg	0.725 mg

Endotoxin assay

All the positive and negative controls provided with the kit provided results in the range suggested by the manufacturer. The Tat-GST (2 mg/mL) recombinant protein contained 16 U/mg and a CA/Tat-GST (2 mg/mL) polyprotein contained 4 EU/mg.

Cytotoxicity of purified protein preparations

VERO cells exposed to 0.1-20 µg protein/well, a purified CA-GST preparation caused a decrease in number of viable cells from 47,860 (GST control) to 33,150 cells/well (containing recombinant protein) whilst a CA/Tat-GST polyprotein preparation resulted in an increase in number of viable cells from 47,860 (GST control) to 108,670 cells/well (exposed to polyprotein) (Figure 4.5). In primary FBL cells exposed to 20 mg protein/well, the JDV CA-GST caused an increase in numbers of viable cells 47,860 (GST control) to 66,430 (containing recombinant protein) whilst the JDV CA/Tat-GST preparation resulted in an increase in viable cell numbers from 68,430 cells/well (GST only) to 128,680 in the wells containing 20 µg of CA/Tat-GST (Figure 4.6).

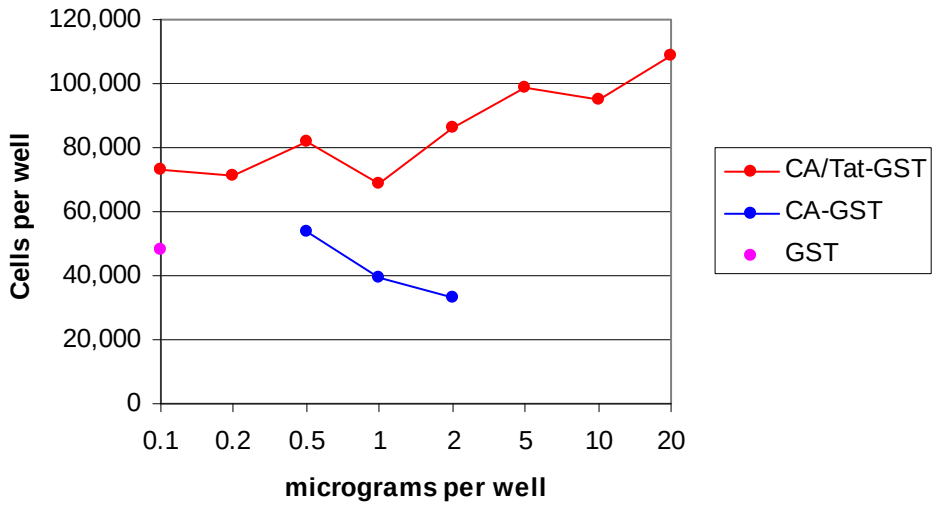


Figure 4.5. Results obtained with a Celltiter96®Aqueous One Solution Cytotoxicity/Proliferation assay using differing amounts of JDV CA/Tat-GST per well over a 4 h period to measure the cytotoxic/proliferative effect of 2 protein preparations in VERO cells (50,000 cells/well) tested for cytotoxicity or proliferation. Results indicated an increase in the cell numbers in the presence of CA/Tat-GST.

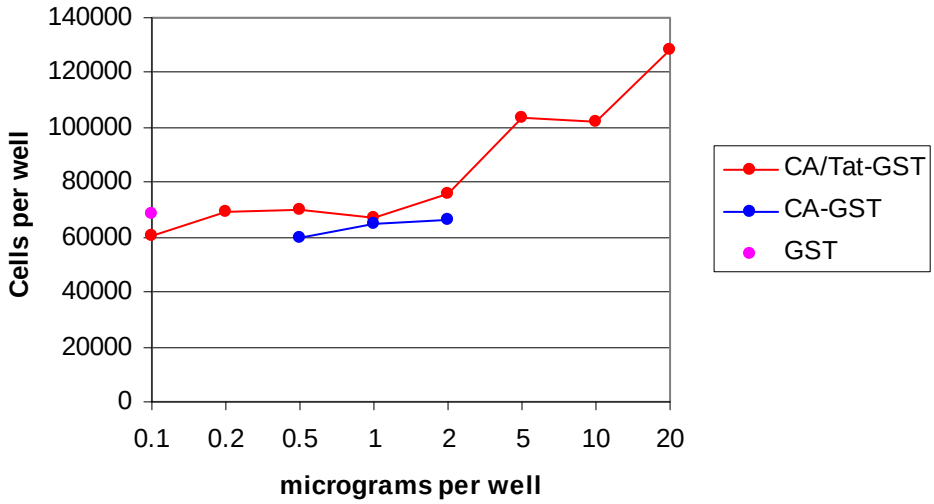


Figure 4.6. Results obtained with a Celltier96®Aqueous One Solution Cytotoxicity/Proliferation assay using differing amounts of JDV CA/Tat-GST polyprotein/well over a 4 h period to measure the cytotoxic/proliferative effect of 2 protein preparations in primary FBL cells (50,000 cells/well) tested for cytotoxicity or proliferation. Results indicated an increase in the cell numbers in the presence of the JDV CA/Tat-GST polyprotein.

Presence of DNA in purified protein preparations

A purified JDV CA/Tat-GST preparation diluted to contain 4 mg protein/mL contained 81.7 ng of DNA/µl, determined using the NanoDrop® ND-1000 Biolab for DNA quantification. A high level of plasmid contamination was detected by PCR in all dilutions (Figure 4.7).

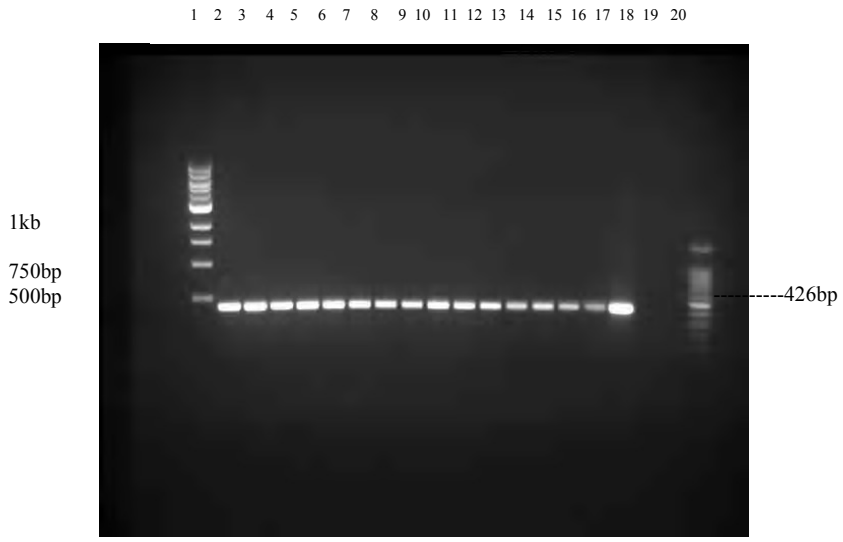


Figure 4.7. Conventional PCR using j604f and j1030r primers (Table 4.1) to detect plasmid DNA in a purified CA/Tat-GST polyprotein preparation. Lane 1, 1 Kb DNA ladder; lane 2, 1:2 dilution of preparation; lane 3, 1:4 dilution; lane 4, 1:8 dilution; lane 5, 1:16 dilution; lane 6, 1:32 dilution; lane 7, 1:64 dilution; lane 8, 1:128 dilution; lane 9, 1:256 dilution; lane 9, 1:512 dilution; lane 10, 1:1,024 dilution; lane 11, 1:2,048 dilution; lane 12, 1:4,096 dilution;, lane 13, 1:8,192 dilution; lane 14, 1:16,384 dilution; lane 15, 1:32,768 dilution; lane 16, positive control (clone #139); lane 18, negative control with no DNA; lane 19, negative control with water only; lane 20, 100 bp DNA ladder. The positive bands of the correct size indicate that there was a high level of plasmid in the vaccine preparations.

Benzonase degradation of free nucleic acid in purified polyprotein preparation

The addition of 75 U/mL of benzonase at room temperature for 45 min but not 15 or 30 min degraded the majority of the DNA and RNA in the purified polyprotein preparation (Figure 4.8). No detectable plasmid DNA was amplifiable by PCR after benzonase treatment of the vaccine preparation.

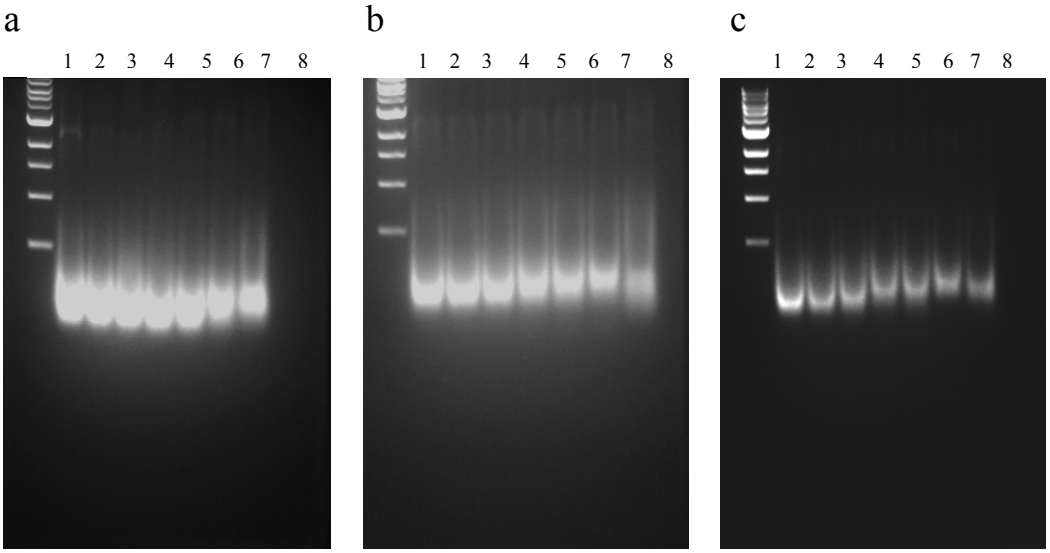


Figure 4.8. Agarose gel electrophoresis to determine the effect of benzonase on DNA/RNA present in a 20 µg of JDV CA/Tat-GST polyprotein preparation. (a) Differing concentrations of benzonase at room temperature for 15 min; (b) differing concentrations of benzonase at room temperature for 30 min; (c) differing concentrations of benzonase at room temperature for 45 min. In each gel, lane 1 contained 50 U/mL of benzonase, lane 2 contained 55 U U/mL of benzonase, lane 3 contained 60 U/mL of benzonase, lane 4 contained 75 U/mL of benzonase, lane 5 contained 100 U/mL of benzonase, lane 6 contained 150 U/mL of benzonase, lane 7 contained 500 U/mL of benzonase, lane 8 was a negative control with no substrate. Treatment with benzonase for 15 and 30 min (gels a and b) resulted in only partial degradation of the nucleic acids at all concentrations of benzonase but treatment for 45 min (gel c) shows that 75 U/mL of benzonase destroyed most of the nucleic acid present in the purified polyprotein preparation.

Stability of the polyprotein at 4ºC

The JDV CA-GST and CA-GST proteins with or without an additional 5% glycerol did not degrade over a 12 month period at 4°C. The Tat-GST was more unstable and there was a reduction of 0.759 mg/mL of the original protein content whilst the Tat-GST plus 5% glycerol had a reduction of 0.4287 mg/mL over the same period. The CA/Tat-GST polyprotein was relatively stable for 12 months both with and without 5% glycerol, with an overall reduction of 0.2783 mg/mL without glycerol and 0.238 mg/mL with 5% glycerol (Figure 4.9).

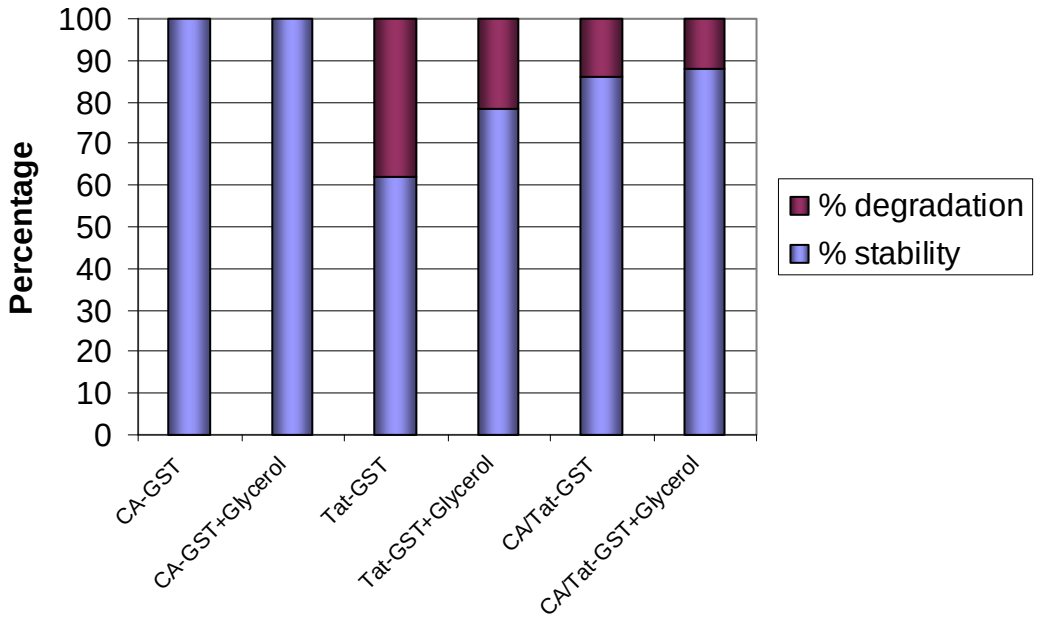


Figure 4.9. A comparison of the stability of recombinant protein preparations at 4ºC over a 12 month period. All recombinant proteins were diluted to 2 mg/mL at the start of the experiment with or without 5% glycerol and their concentration measured 12 months later. The CA-GST was the most stable recombinant protein whilst the Tat-GST was the least stable.

Discussion

A prokaryotic expression system was chosen for the production of a JDV recombinant vaccine as this was a technology considered feasible in Indonesia. The vector system chosen was the pGEX6-P-1 GST plasmid vector system (Amersham) which incorporates the 26 kDa GST protein of the *Schistosoma japonicum* to the recombinant proteins of interest. This system had been previously used for the production of other JDV proteins (Burkala et al., 1998; Burkala et al., 1995) and earlier JDV vaccines (Ditcham, 2007) with success. The advantages of this system were that the recombinant proteins are often folded into inclusion bodies which make the purification simpler and less expensive than other systems, some of which require specialized columns for affinity purification (LaVallie and McCoy, 1995; Yin et al., 2007). In addition, the GST tag is derived from *S. japonicum* which produces a zoonotic disease that is endemic in many areas of Indonesia (Chitsulo et al., 2000;

Engels et al., 2002). Vaccination of Chinese cattle in endemic regions with the 26 kDa GST protein was reported to reduce worm burden by up to 30% (Wu et al., 2004). This effect is a potentially significant advantage as the vaccine may not only protect against JDV but may also offer some protection against *S. japonicum*. A disadvantage of the pGEX6-P-1 (GST) system is that it does not differentiate infected from vaccinated cattle as cattle naturally infected with *S. japonicum* will have antibodies against the GST. A possible way to differentiate naturally-infected from vaccinated cattle would be to detect JDV Tat antibodies as these are not normally present in naturally infected cattle (Setiyaningsih et al., 2008).

The recombinant proteins used in this Chapter were purified from the insoluble inclusion bodies that were washed with varying concentrations of Triton X-100 and then solubilised in a urea, DTT and Tris based solution (Kopito, 2000). Soluble proteins were also produced, ranging from 20-50% of the total protein produced by the *E. coli* but these were not purified and used due to the likely costs involved (Ditcham, 2007). These bacterial inclusion bodies are refractile aggregates of protease-resistant misfolded protein that occur when there is an over-expression of a protein in the cell (Carrio and Villaverde, 2002). The excess of protein produced in relation to various chaperone proteins leads to the protein being mis-folded and stored in an aggregate within the cell (Carrio and Villaverde, 2001; Schwarz et al., 1996). This inclusion body formation allows the bacteria to store protein for prolonged periods of time which can then be refolded correctly into an active form at a later date when needed by the bacterium (Carrio et al., 1999; Carrio and Villaverde, 2001). The purification of recombinant proteins from inclusion bodies offers many advantages, including ease of purification, high purity of the protein present in the inclusion bodies and the resistance of inclusion bodies to proteases, temperature and pH changes (Carrio and Villaverde, 2002). The main disadvantage of purifying protein from inclusion bodies is that sometimes the protein can be incorrectly folded which leads to a loss of conformational epitopes or loss of activity in the case of enzymes. These inclusion bodies can also be toxic to the bacterial cells (Gonzalez-Montalban et al., 2005). It is usually important to correctly re-fold the protein after purification from inclusion bodies to retain the activity and the binding properties (Lilie et al., 1998) but particularly for Tat a loss of enzymatic function was desirable due to the likely deleterious effects of Tat on the vaccinated cattle. Furthermore, a study using a

soluble and a urea/dithiothreitol (DTT) solubilised recombinant *Taenia ovis* vaccine (45W-GST) found increased immunogenicity and protection using the urea/DTT solubilised inclusion body vaccine compared to the native soluble vaccine (Rothel et al., 1997).

Several methods of cell lysis were shown to be effective and to release the inclusion bodies from bacterial cells. Triton X-100 treatments achieved similar results to sonication and freeze/thawing, demonstrating that a chemical means of lysis was feasible. However, numerous factors can affect chemical lysis, such as incomplete agitation, temperature, time and incomplete mixing of the lysis buffer and most large scale lysis protocols use sonication, a French press or freeze/thawing as a method of cell lysis (Feliu et al., 1998).

Considerable amounts of nucleic acid were detected in the purified protein preparations produced, consisting of plasmid DNA which was demonstrated to be present and presumably also bacterial RNA and DNA. This was degraded by the addition of benzonase to the lysis buffer. The optimum concentration of benzonase for degradation was 75 U/ mL for 45 min which is similar to the recommended working concentration of 50 U/ mL for 10 min (Novagen) and concentrations reported in previous publications (Friedhoff et al., 1996; Meiss et al., 1995).

Protein yields were quantified using high quality gel imaging densitometry (ProExpress, PerkinElmer) rather than by biochemical protein assay (Bradford, BioRad) so that only the protein of interest was quantified rather than the total protein content. This allowed accurate quantification of the protein of interest and the percentage purity of the final protein preparation to be determined.

Endotoxin contamination was detected in the purified protein preparations using a Limulus Amoebocyte lysate (LAL) assay (Levin and Bang, 1964; Poole et al., 2003; Tomasulo et al., 1977). This assay is one of 2 assays commonly used to measure endotoxin levels in vaccine preparations, the other being a rabbit pyrogen test which measures endotoxin but also other pyrogens. Recently, one-plate assays for pyrogen detection have also been developed but these are expensive and were unavailable for this current study (Gaines Das et al., 2004). The level of endotoxin detected was 8-fold greater in the individual JDV recombinant CA-GST and Tat-GST preparations than the fused JDV CA/Tat-GST polyprotein/mg of protein obtained. This was

expected as there is a greater yield of the polyprotein/cell and a higher purity of the final polyprotein preparation compared to the individual recombinant protein vaccine.

It is important that endotoxin contamination should be detected in any protein preparation intended for injection. They are a lipopolysaccharide (LPS) complex that form part of the outer membrane of Gram-negative bacteria such as *Salmonella, Pseudomonas, Shigella and E. coli* (Bennett and Cluff, 1957). The majority of these endotoxins are released when the bacterial cells are lysed and they are extremely difficult to remove or inactivate and remain in the environment for prolonged periods making them a medically important contaminant of protein preparations (Poole et al., 2003). In ruminants, endotoxins induce increased blood lactate, hyperglycaemia, leucopenia followed by granulocytosis, and disseminated intravascular coagulation which can lead to endotoxic shock and death. Gross pathological lesions of the heart, brain, adrenals and lungs are also commonly associated with endotoxic exposure (Nagaraja et al., 1979). This endotoxic shock followed by a later second exposure to endotoxin will produce an anaphylactic response in ruminants. Safe endotoxin levels in proteins for injection in ruminants have not been determined but they are probably equivalent to levels in human vaccines which are 5 EU/kg parenterally or 0.2 EU intrathecally (Magalhaes et al., 2007).

Despite the presence of endotoxins in all the purified protein preparations, not all were cytotoxic as determined by a cytotoxicity/proliferation assay in VERO and primary FBL cells. The GST and the CA-GST appeared to have little cytotoxic or proliferative effect on either VERO or primary FBL cells. The addition of JDV CA/Tat-GST polyprotein actually led to hyper-proliferation of these cells. This proliferation may have been due to the JDV Tat component of the vaccine as HIV Tat has been shown to induce abnormal levels of cellular cytokines in infected cells leading to cell hyper-proliferation in a dose dependant manner (Albini et al., 1998; Badou et al., 2000; Conaldi et al., 2002; Vellutini et al., 1995) although BIV Tat has been reported to have an apoptotic effect *in vitro* (Xuan et al., 2008). Since the CA/Tat polyprotein was used as a vaccine (Chapters 5 and 6), the possible proliferative effect of the Tat component needs further investigation to determine if the polyprotein would need to be further modified by oxidization or mutated to remove all Tat activity whilst retaining immunogenicity, as has been attempted with HIV Tat (Bennasser et al., 2002; Caselli et al., 1999; Noonan et al., 2003).

With the exception of Tat-GST all of the proteins produced, including the CA/Tat-GST polyprotein had less than 25% degradation when held at 4°C for 12 months. Glycerol, which can act as a stabilising agent for enzyme activity and protein conformation (Gekko and Timasheff, 1981) had no significant effect on the stability except for the Tat-GST protein individually. While the degradation that was observed would need to be factored into the dose used if the polyprotein was used as a vaccine, the stability at 4ºC would make it suitable were it to gain acceptance as a vaccine in Indonesia.

In conclusion, the successful production of JDV CA/Tat-GST polyprotein in a single fermentation reaction would decrease costs of production and enable higher purity and lower levels of endotoxin contamination to be achieved than were obtained with the individual CA-GST and Tat-GST preparations. Nucleic acid contamination of the proteins was detected but could be removed by the addition of benzonase, which would not add prohibitive additional costs to the purification process. Further work will need to be undertaken to determine the stability and potency of the polyprotein if it is used for a vaccine and the proliferative effect of the protein noted *in vitro* should also be investigated further. The Tat portion of the vaccine should also be tested to determine the level of activity present. If necessary, methods of inactivation of the Tat component without affecting its immunogenicity and antigenicity should be investigated.

Chapter 5. Response of cattle to a recombinant CA/Tat-GST polyprotein Jembrana disease vaccine

Summary

This chapter describes the use of bacterial expressed recombinant JDV CA/Tat-GST polyprotein as a vaccine and a comparison with a vaccine containing a combination of individual CA-GST and Tat-GST recombinant proteins. Groups of cattle were vaccinated twice and then challenged with a pathogenic JDV_{Pul01} strain. Vaccination elicited very high titres of CA and Tat antibody of IgG, IgM and IgA subclasses prior to challenge with JDV. The vaccine ameliorated the hyperpyrexia and severe lymphopenia typically seen in cattle infected with JDV but did not provide complete protection.

Introduction

The current vaccine used in Indonesia for Jembrana disease is a whole virus tissue-derived inactivated virus vaccine prepared from spleen tissue obtained from cattle experimentally infected with JDV. The spleen is homogenized and treated with Triton X-100 to inactivate the virus, then emulsified in a mineral oil adjuvant (Hartaningsih et al., 2001). This vaccine has been demonstrated to provide partial protection, ameliorating the disease process in animals challenged after vaccination, but not preventing infection (Hartaningsih et al., 2001). This vaccine is not only expensive to produce but its use is associated with safety and ethical issues (Hartaningsih et al., 2001; Hartaningsih et al., 1995a). Alternative vaccines consisting of recombinant JDV proteins, including CA, SU, TM and Tat, have been expressed with a glutathione-S-transferase tag (GST) in *E. coli* and tested for efficacy as vaccines. A vaccine preparation consisting of 2 mg of CA-GST plus 2 mg of Tat-GST in an oil adjuvant and administered 2-3 times was selected as the most promising recombinant protein vaccine, although again protection was limited to amelioration of the disease process after infection (Ditcham, 2007).

As the production of 2 separate recombinant protein antigens was expected to increase the difficulty of producing a vaccine based on these proteins, a single plasmid construct expressing both CA and Tat as a single GST-tagged polyprotein (JDV CA/Tat-GST) was developed (Chapter 4). In this Chapter, the polyprotein was

prepared as a vaccine and tested for efficacy in small groups of cattle. The efficacy of the CA/Tat-GST polyprotein was compared with the administration of 2 single JDV protein preparations, CA-GST and Tat-GST.

Materials and methods

Preparation of recombinant proteins as vaccines

Individual JDV CA-GST and JDV Tat-GST recombinant proteins were produced and purified as described previously (Chapter 4) and adjusted to a concentration of 4 mg/mL in PBS (pH 7.4). The purity of the proteins is demonstrated in Figure 5.1. Western immunoblots were performed to confirm that the recombinant polyproteins were recognized by antibody from naturally infected, conventionally vaccinated and CA/Tat-GST vaccinated cattle sera as previously described (Chapter 4). The individual protein suspensions were mixed in equal volumes and then emulsified with an equal volume of incomplete Freund's adjuvant (IFA) and a dose of 2 mL (containing 2 mg of each GST-tagged protein) was administered to cattle.

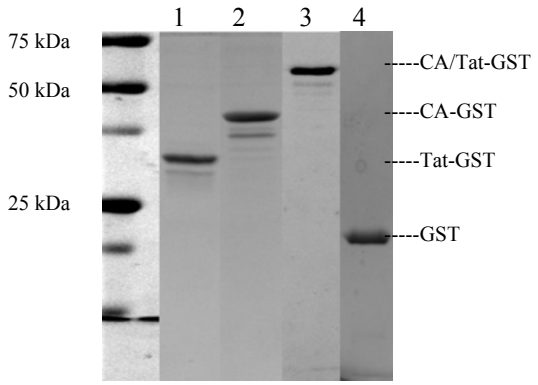


Figure 5.1. Coomassie Brilliant Blue stained SDS-PAGE gel showing the purity of the recombinant proteins used for vaccination. Lane 1, Tat-GST; lane 2, CA-GST; lane 3, CA-Tat-GST; lane 4, GST only. The gel demonstrates the purity of the GST preparation and >90% purity of the other recombinant proteins.

The JDV CA/Tat-GST recombinant polyprotein was produced as previously described (Chapter 4). Briefly, JDV CA/Tat-GST transformed BL21 *E. coli* were grown to an OD_{600} of 1.5 in 2YT medium plus 1 mM ampicillin before induction of expression by the addition of 0.1 mM IPTG (final concentration) for 12 h. The cells were then lysed by sonication and centrifuged. The fusion protein from the lysed pellet was in an insoluble form, and was further purified by 6 wash steps (1 h each on a rotary mixer at 4ºC) with a 0.5-2% Triton X-100 wash buffer (Tris-HCl 10 mM, EDTA 5 mM and

0.5-2% [w/v] Triton X-100) followed by centrifugation; the first 2 washes contained 2% Triton X-100, the next 2 washes contained 1% Triton X-100 and the final 2 washes 0.5% Triton X-100. The protein in the final pellet was then solubilised overnight after the addition of a urea/Tris solution (urea 2 M, Tris 2 M, DTT 20 mM and PMSF 1 mM). The purified antigens were analysed by SDS-PAGE and Coomassie Brilliant Blue staining to determine yield and purity as previously described (Chapter 4) and the purity of the final protein used is demonstrated in Figure 5.1. The proteins were then diluted in PBS (pH 7.4) to the desired concentration and mixed with adjuvant for use as the vaccine. Western immunoblots were also performed to confirm that the recombinant polyprotein was recognized by antibody from naturally infected, conventionally vaccinated and CA/Tat-GST vaccinated cattle sera as described in Chapter 4. The protein suspension was adjusted to a concentration of 4 mg/mL, emulsified with an equal volume of IFA, and a dose of 2 mL (containing 4 mg of GST-tagged polyprotein) was administered to cattle.

GST for mock vaccination of cattle was prepared from BL21 *E. coli* containing the pGEX6-P-1 plasmid with no JDV inserts. The plasmid-containing *E. coli* were grown in 2YT broth supplemented with 1 mM ampicillin to an OD_{600} of 0.6. Expression of the recombinant protein was then induced with IPTG (0.1 mM final concentration) for 4 h. The bacteria were then harvested by centrifugation, washed in PBS, resuspended in 25 mL of lysis buffer and lysed by sonication. After clarification of the lysate by centrifugation, the supernatant was passed 3 times through a glutathione/Sepharose resin column of 0.2 mL, equilibrated with PBS. Soluble bound GST protein was then displaced with 10 column volumes of 30 mM glutathione in PBS. The eluted GST was then tested for purity by SDS-PAGE (Figure 5.1) and quantified by densitometry as previously described (Chapter 4). The protein suspension was adjusted to a concentration of 2 mg/mL, emulsified with an equal volume of IFA, and a dose of 2 mL (containing 2 mg of GST) was administered to cattle.

Preparation of non-GST tagged proteins as antigens for analysis of antibody responses to vaccine antigens

Histidine (his)-tagged CA was produced as previously described (Chapter 3).

Biotin-tagged Tat (Tat-biotin) was produced with a Pinpoint (Promega) recombinant plasmid clone jTat1, previously constructed by Dr Moira Desport (this laboratory),

containing JDV *tat*1 from nucleotide positions 5,010-5,303 of the genome of JDV_{Tab87} (Chadwick et al., 1995a); this plasmid was predicted to encode a JDV Tat1 protein 92 amino acids in length. Optimum expression of Tat was achieved by adding a 1:50 volume of an overnight culture of *E. coli* in 2YT broth plus 1 mM ampicillin to 1 L of 2YT broth plus 1 mM ampicillin. The culture was grown to an OD_{600} of 0.6 before induction of expression by the addition of IPTG (0.1 mM final concentration) for 4 h, and the cells were then pelleted by centrifugation (10,000 g for 15 min at 4°C). The recombinant protein was produced in inclusion bodies in an insoluble form. The cells were resuspended in cell lysis buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl and 5 % [v/v] glycerol) and lysed by sonication. After centrifugation (10,000 g for 15 min) the cell pellet containing the inclusion bodies was resuspended in 10 mL of PBS (pH 7 adjusted with HCl) and agitated overnight at 4°C on an orbital mixer. The cell pellet was collected by centrifugation (10,000 g for 15 min at 4°C). The pellet was washed for 10-30 min in PBS (pH 7) at 4°C and centrifuged (10,000 g for 15 min at 4°C), repeating this wash step 4 times. The protein in the final pellet was solubilised by the addition of 9 mL of solubilisation buffer (urea 2 M, Tris 2 M, DTT 20 mM and PMSF 1 mM) on an orbital mixer at 4°C for 2 h. The solubilised pellet was added slowly to 390 mL of Refolding Buffer (Tris pH 8 100 mM, 350 mg CHAPS and 0.53 g DTT made up to 350 mL with deionised water) and the refolded protein was concentrated in an ultrafiltration cell (Amicon) until a volume of 20 mL was obtained. The final protein was filtered using a standard 0.22 μ syringe exclusion filter (Millipore) to remove aggregated *E. coli* proteins and then dispensed in 1 mL amounts and stored at -80°C. These individual non GST-tagged proteins were used to determine the protein specific antibody titres induced by the vaccine.

Vaccination trial

Twenty female 12-month-old Bali cattle were obtained from Nusa Penida, an island adjacent to Bali where Jembrana disease does not occur, transported to Bali, housed in insect-free animal houses, and maintained as previously described (Soeharsono et al., 1990). On arrival, the cattle were vaccinated with Haemorrhagic Septicaemia vaccine (PUSVETMA) and confirmed as being JDV antibody-negative by ELISA and Western immunoblotting, and housed for 7 days prior to further use to adapt to the conditions.

All animals were tested prior to challenge for serum antibody to the vaccine proteins by Western immunoblot and ELISA. Briefly, 2,000 ng of Tat-biotin and CA-his, were subjected to SDS-PAGE and transferred to nitrocellulose membrane using standard methods. The membranes were blocked in 5% skimmed milk powder in TBST. Nitrocellulose strips were reacted with a 1:100 dilution of sera for 1 h with constant rocking, and then washed in TBST. Bound bovine IgG was detected with HRP conjugated anti-bovine IgG (1:1,000 dilution in TBST) and excess conjugated antibody was removed by washing in TBST. The presence of specific antibody to the JDV proteins was revealed by reaction with the chromogenic substrate 4-chloronaphthol (BioRad; prepared as a 1 mg/mL solution in methanol and diluted 1:5 in TBS with an additional 1 μL/mL of 30 % H_2O_2).

The 20 cattle were divided into 4 groups of 5: groups A and D received 2 vaccinations at days 28 and 56 whilst groups B and C had 3 vaccinations at days 0, 28 and 56. Group A was vaccinated twice, at day 28 and 56, each time with 4 mg of the fused polyprotein vaccine in IFA. Group B was vaccinated 3 times at days 0, 28 and 56 with 4 mg of the fused polyprotein in IFA. Group C was vaccinated 3 times at days 0, 28 and 56 with a mix of 2 mg of CA-GST and 2 mg of Tat-GST per vaccination in IFA. Group D (controls) were vaccinated 3 times with 2 mg GST in IFA.

Table 5.1. Groups of cattle inoculated with recombinant purified proteins emulsified in IFA and subsequently challenged 28 days after the final vaccine dose with 1,000 ID_{50} of JDV_{Pul01}.

Group and number (n) of cattle	Vaccine	Dose (mg)	Number of doses
Group A (n=5)	CA/Tat-GST	4 mg	2
Group B (n=5)	CA/Tat-GST	4 mg	3
Group C (n=5)	Tat-GST + CA-GST	2 mg + 2 mg	3
Group D (n=5)	GST	2 mg	2

All cattle were challenged with 1,000 50% infectious doses (ID_{50}) of the heterologous JDV_{Pul01} on day 84 of the trial, 28 days after the final vaccine dose (Table 5.1) as

described by Soeharsono et al. (1990). The infectious JDV_{Pul01} was obtained from additional JDV antibody-negative cattle that had been inoculated with JDV_{Pul01} as previously described (Soeharsono et al., 1990). Briefly, on the second day of the febrile reaction after inoculation with JDV_{Pul01}, heparinized blood was collected from the these additional cattle and the approximate ID_{50} of virus in the blood determined indirectly by antigen capture ELISA as previously described (Stewart et al., 2005) in order to calculate the dilution required to provide a challenge dose of 1,000 ID_{50}. Viral RNA was extracted from plasma samples taken from the donor animals and virus RNA quantified by qRT-PCR (see below) to retrospectively confirm the estimated infectious dose (Stewart et al., 2005).

Following inoculation of the vaccinated and control cattle with JDV_{Pul01}, serum samples were collected from all cattle at the times shown in Table 5.2. Total leucocyte counts were performed at times shown in Table 5.2. Blood smears were prepared and used to determine the differential leucocyte response; these smears were dried for 15 min before being stained with Diff-Quik differential leucocyte stain (Difco). Two hundred leucocytes were examined per slide to determine the differential leucocyte response. Total leucocyte counts were also performed on the same days using heparinised blood samples as described previously (Soeharsono *et al* 1995) and rectal temperatures were taken from days 0-110 of the trial and a rectal temperature $\geq$ 39.3ºC used to define the occurrence of fever. Aspects of the febrile response, including the time for onset of fever (>39.3ºC) after infection, the mean febrile temperature during the febrile response, the maximum febrile temperature and the duration of fever with low rectal temperatures (39.3-40.2ºC), moderate rectal temperature (40.3-41.2ºC) and high rectal temperatures (hyperpyrexia) of >41.3ºC, were calculated. Fever score was used as a measure of the severity of the febrile period, and calculated as the area under the body temperature curve, but above the 39.3°C cut-off line (Ditcham, 2007). Group data was analysed using a Students 2 sample t-test to identify significant differences.

Table 5.2. Schedule for the recombinant protein vaccine trial, showing the time of vaccination, time of subsequent challenge with JDV and the sampling dates.

Day No	Vaccination and/or challenge with JDV	Days post-challenge with JDV	Sample collected/examination				
			Plasma	Serum	PBMC	Rectal temp	WBC count
-7			X	X	X		
-1							
0	1st vaccination group B and C		X	X	X	X	X
7				X		X	
14				X		X	
21				X		X	X
28	1st vaccination group A and D			X		X	X
28	2nd vaccination groups B and C					X	
35				X		X	X
42				X			
49				X			
56	2nd vaccination groups A and D			X			
56	3rd vaccination group B and C						
63				X			
70				X			
77				X			
84	Challenged with JDV	0	X	X	X	X	X
85		1	X		X	X	X
86		2	X		X	X	X
87		3	X		X	X	X
88		4	X		X	X	X
89		5	X		X	X	X
90		6	X		X	X	X
91		7	X	X	X	X	X
92		8	X		X	X	X
93		9	X		X	X	X
94		10	X		X	X	X
95		11	X		X	X	X
96		12	X		X	X	X
97		13	X		X	X	X
98		14	X	X	X	X	X
99		15	X		X	X	X
100		16	X		X	X	X
101		17	X		X	X	X
102		18	X		X	X	X
103		19				X	
104		20	X		X	X	
105		21				X	
106		22	X	X	X	X	
107		23				X	
108		24	X		X	X	
109		25				X	
110		26	X	X	X	X	

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) to determine plasma virus load

RNA was extracted from plasma samples which were centrifuged (8,000 g for 5 min) prior to extraction of RNA. Total RNA from 140 µL of plasma was extracted using a QIAmp Viral RNA mini kit (QIAGEN) as recommended by the manufacturer. Extracted RNA was stored at –80^{o}C until required.

Primer and probe sequences (Table 5.3) for the qRT-PCR were designed to amplify a 121 bp fragment of the JDV *gag* and were derived from the previously published

JDV_{Tab87} sequence (Genbank accession No. U21603; (Chadwick et al., 1995c). The protocol for the qRT-PCR was based on the method described by Stewart et al. (2005).

Table 5.3. Primers and probe used for the qRT-PCR for the determination of viral load from plasma of the experimentally challenged cattle.

Primer	Sequence	Nucleotide position in JDV_{Tab87} genome
JDV gag1f	5'-GGGAGACCCGTCAGATGTGGA-3'	536-555
JDV gag1r	5'-TGGGAAGCATGGACAATCAG-3'	635-654
Probe	FAM-5'-CCCACAACTTAGAAAGAACTTCCCCGCTG-3'-BHQ-1	604-632

The fluorogenic probe (Geneworks) was labelled at the 5' end with the reporter dye, 6-carboxyfluorescein, and at the 3' end with the Black Hole-2 quencher dye. A premixed 2X mastermix (Iscript; BioRad) was used, with the addition of both primers and fluorogenic probe to a final concentration of 100 nM, and ultra pure water added to give a final 1X concentration. MMLV reverse transcriptase was used in this assay following the manufacturer's instructions. One µL of RNA sample was added to 9 µL of master mix in 100 µL tubes (Axygen) and the one-step RT-PCR protocol consisted of an RT step of 50°C for 10 min, a 5 min inactivation step at 95°C with a 2 second pause at 92°C to prevent thermal overshoot, followed by 40 cycles of 92°C for 2 seconds, 95°C for 15 seconds, 58°C for 30 seconds.

A standard curve was generated in each run by amplification of duplicate serial 10-fold dilutions of plasmid pCR2.1 (Invitrogen) containing nucleotides 19-2881 of the sequence of the JDV_{Tab87} encompassing the target amplicon. Copy numbers of the plasmid added to each standard reaction were calculated, with 1 copy of plasmid equivalent to 1 viral genome. Estimation of the relative efficiency of the RT step was performed by including a standard RNA sample and blanks (no probe, no template, no RT, template but no RT) in each run. The results were analysed using Rotor-Gene proprietary software Version 6.0. Group data was analysed using a Students 2 sample t-test to identify significant differences between groups and between vaccinated and control cattle.

Serological tests to determine response to CA and Tat in vaccinated cattle

Western immunoblotting was performed as described above but with biotin-tagged JDV Tat and his-tagged JDV CA which was produced as described before.

Antibody titres were determined by ELISA using Maxisorb™ plates (Nunc) coated with 200 ng/well of his-tagged JDV CA (4 µg/mL in 0.1 M carbonate buffer pH 9.6). These plates were incubated overnight at 4ºC then washed twice in PBST (0.05 %) and blocked with 5 % skimmed milk powder in PBST at room temperature for 30 min. A starting dilution of 1:20 was then added to the first wells diluted in PBST with 5 % skimmed milk powder. This was then incubated for 1 h at 37ºC then washed 3 times with PBST (0.05 %) and 100 µL of anti-bovine IgG (ICN), IgM (Bethyl) or IgA (Bethyl) conjugated to HRP diluted in skimmed milk PBST at a dilution of 1:2,000 and incubated for 1 h at 37°C. These plates were then washed once in PBST (0.05%) then twice in PBS (pH 7.4). A HRP colour development reagent (BioRad) was then added for 15 min and the reaction was then stopped with 2% oxalic acid solution. The plates were read at OD_{405} and the endpoint titre was determined as the reciprocal of the last dilution with an OD_{405} more than 3 times that of sera from the same animal collected at day 0. By definition, therefore, samples collected at day 0 had no protein-specific antibody titre.

Group serological data was analysed using a Students 2 sample t-test to identify significant differences.

Results

Humoral immune response to vaccine proteins

IgG response to vaccination

The cattle vaccinated 3 times with the recombinant JDV CA/Tat-GST polyprotein (Group B) and those vaccinated only twice with the same preparation all developed high titres of JDV CA and Tat antibody. The titres of antibody to the CA protein were greater than those to the Tat protein. The CA and Tat antibody titres were higher in those vaccinated 3 times with the polyprotein (Group B) than those vaccinated 2 times with the polyprotein (Group A). The 2 groups of cattle vaccinated with the JDV CA/Tat polyprotein (Groups A and B receiving 2 and 3 vaccine doses, respectively) had higher CA and Tat antibody titres than those cattle vaccinated 3 times with the

mixture of individual JDV CA-GST and JDV Tat-GST proteins (Group C) (Table 5.4 and Figure 5.2).

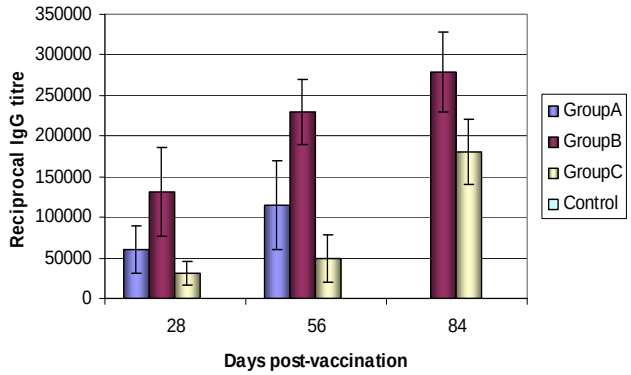
Table 5.4. The vaccine specific IgG antibody responses against the JDV CA and Tat antigens at days 28, 56 and day 84 (day of challenge) in groups A, B and C. The antibody titres were determined as the reciprocal of the last dilution with an OD_{405} more than 3 times that of sera from the same animal collected at day 0. By definition, therefore, samples collected at day 0 had no protein-specific antibody titre.

Group and animal	Antigen	Days after initial vaccination			Mortality
		28	56	84	
Group A (2X CA/Tat-GST)					
CB139	CA	1:163,840	>1:327,680	NT	Survived
	Tat	1:160	1:5,120	NT	
CB140	CA	1:20,480	1:81,920	NT	Died
	Tat	<1:20	1:2,560	NT	
CB141	CA	1:81,920	1:81,920	NT	Survived
	Tat	1:320	1;1280	NT	
CB142	CA	1:10,240	1:40,960	NT	Survived
	Tat	1:20	1:2,560	NT	
CB143	CA	1:20,480	1:40,960	NT	Survived
	Tat	<1:20	1:2,560	NT	
Group B (3X CA/Tat-GST)					
CB144	CA	1:40,960	1:163,840	1:81,920	Survived
	Tat	<1:20	1:640	1:2,560	
CB145	CA	>1:327,680	>1:327,680	>1:327,680	Survived
	Tat	1:5,120	1:1,280	1:10,240	
CB146	CA	1:163,840	1:163,840	>1:327,680	Survived
	Tat	<1:20	<1:20	<1:20	
CB147	CA	1:40,960	>1:327,680	>1:327,680	Survived
	Tat	1:160	1:5,120	1:20,480	
CB148	CA	1:81,920	1:163,840	>1:327,680	Survived
	Tat	<1:20	1:320	1:2,560	
Group C (3X CA-GST + Tat-GST					
CB149	CA	1:20,480	1:20,480	1:81,920	Survived
	Tat	1:160	1:80	1:1,280	
CB150	CA	1:40,960	1:40,960	1:163,840	Survived
	Tat	1:640	1:320	1:1,280	
CB151	CA	1:81,920	1:163,840	>1:327,680	Survived
	Tat	1:1,280	1:1,280	1:1,280	

CB152	CA	1:10,240	1:10,240	1:163,840	Died
	Tat	1:2,560	1:1,280	1:1,280	
CB153	CA	1:640	1:10,240	1:163,840	Survived
	Tat	<1:20	<1:20	1:640	

NT denotes not tested

a



b

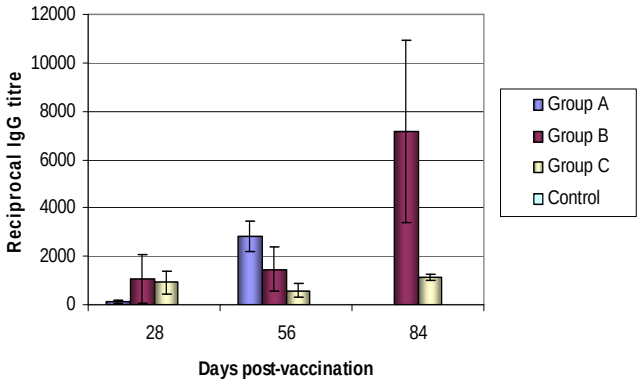


Figure 5.2. Graphical representation of the protein-specific IgG titres ± SE to the JDV CA and Tat antigens at different time prior to challenge with JDV. (**a**) JDV anti-CA IgG titres; (**b**) JDV anti-Tat specific IgG titres. The cattle inoculated with the CA/Tat-GST polyprotein had higher IgG anti-CA responses at all time points and higher IgG anti-Tat by the day of challenge (day 84) than did those cattle inoculated with the individual recombinant proteins. The cattle inoculated 3 times with the CA/Tat-GST polyprotein had significantly higher ($p=0.00334$) anti-CA specific titres at day 56 and approached significance at day 28 and 84 ($p=0.055426$ and 0.079964, respectively). The cattle inoculated 3 times with the CA/Tat-GST polyprotein had higher anti-Tat specific titres at all time points than those receiving the individual proteins, and the differences approached significance ($p=0.073527$) at day 84.

IgM response to vaccination

All cattle in Groups A, B and C developed a CA-specific IgM antibody response which was detected 28 days after the initial vaccine dose and continued until the time of virus challenge but a JDV IgM anti-Tat response was detected in one animal only, CB147 in Group B, 84 days after the initial vaccine dose. At day 56 after the initial vaccine dose, the cattle in Group A had a higher mean anti-CA IgM titre than in Group B but those cattle in Group B developed higher mean IgM anti-CA titres 28 days after the third vaccine doses. The cattle vaccinated with the individual JDV CA-GST and JDV Tat-GST (Group C) had similar mean IgM anti-CA titres to those receiving the JDV CA/Tat polyprotein (Table 5.5 and Figure 5.3).

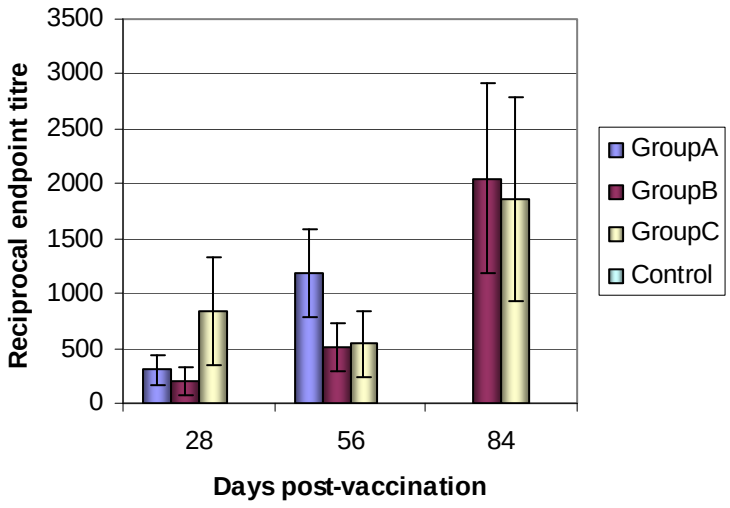


Figure 5.3. Graphical representation of the mean IgM titres ± SE to the JDV CA antigen at different time points prior to challenge with JDV. The CA/Tat-GST vaccinated cattle all had similar IgM anti-CA responses to the CA-GST + Tat-GST vaccinated cattle prior to challenge (day 84).

Table 5.5. The protein specific IgM antibody responses of cattle in groups A, B and C against the JDV CA and Tat antigens at days 28, 56 and day 84 (day of challenge with JDV). The antibody titres were determined as the reciprocal of the last dilution with an OD_{405} more than 3 times that of sera from the same animal collected at day 0. By definition, therefore, samples collected at day 0 had no protein-specific antibody titre.

Group and animal	Antigen	Days after initial vaccination			Clinical outcome
		28	56	84	
Group A (2X CA/Tat-GST)					
CB139	CA	1:640	1:2,560	NT	Survived
	Tat	<1:20	<1:20	NT	
CB140	CA	1:160	1:640	NT	Died
	Tat	<1:20	<1:20	NT	
CB141	CA	1:640	1:1,280	NT	Survived
	Tat	<1:20	<1:20	NT	
CB142	CA	1:20	1:1,280	NT	Survived
	Tat	<1:20	<1:20	NT	
CB143	CA	1:80	1:160	NT	Survived
	Tat	<1:20	<1:20	NT	
Group B (3X CA/Tat-GST)					
CB144	CA	<1:20	1:320	1:1,280	Survived
	Tat	<1:20	<1:20	<1:20	
CB145	CA	1:640	1:640	1:2,560	Survived
	Tat	<1:20	<1:20	<1:20	
CB146	CA	<1:20	<1:20	<1:20	Survived
	Tat	<1:20	<1:20	<1:20	
CB147	CA	1:320	1:1,280	1:5,120	Survived
	Tat	<1:20	<1:20	1:160	
CB148	CA	<1:20	1:320	1:1,280	Survived
	Tat	<1:20	<1:20	<1:20	
Group C (3X CA-GST + Tat-GST					
CB149	CA	<1:20	<1:20	1:320	Survived
	Tat	<1:20	<1:20	<1:20	
CB150	CA	1:320	1:160	1:1,280	Survived
	Tat	<1:20	<1:20	<1:20	
CB151	CA	1:1,280	1:1,280	1:5,120	Survived
	Tat	<1:20	<1:20	<1:20	
CB152	CA	1:2,560	1:1,280	1:2,560	Died
	Tat	<1:20	<1:20	<1:20	
CB153	CA	<1:20	<1:20	<1:20	Survived
	Tat	<1:20	<1:20	<1:20	

NT denotes not tested.

IgA response to vaccination

All 3 groups of cattle immunized with the recombinant CA and Tat proteins developed anti-CA IgA. The titres were highest in cattle vaccinated 3 times with the fused recombinant JDV CA/Tat-GST polyprotein (Group B), less in the cattle vaccinated twice with the fused recombinant JDV CA/Tat-GST (Group A) and least in the cattle receiving the individual JDV CA-GST and JDV Tat-GST proteins (Group C). None of the cattle in any of the groups developed a detectable IgA antibody response to JDV Tat (Table 5.6 and Figure 5.4).

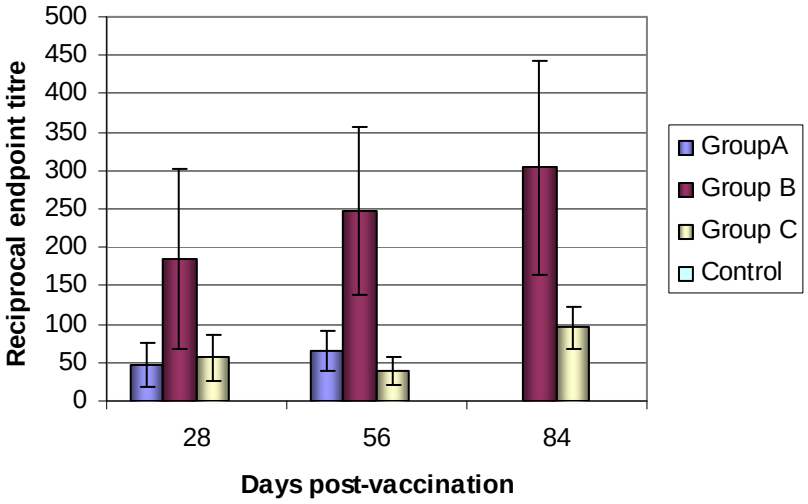


Figure 5.4. Graphical representation of the mean vaccine specific IgA titres ± SE to the JDV CA antigen at different times prior to challenge with JDV. Group A, vaccinated 3 times with the CA/Tat-GST polyprotein had higher IgA anti-CA responses than group C vaccinated with the individual CA-GST and Tat-GST proteins, with levels significantly higher at day 56 (p=0.048356) and approaching significance (p=0.090705) at day 84.

Table 5.6. The protein specific IgA antibody responses of cattle in groups A, B and C against the JDV CA and Tat antigens at days 28, 56 and day 84 (day of challenge with JDV). The antibody titres were determined as the reciprocal of the last dilution of serum with an OD_{405} more than 3 times that of serum from the same animal collected at day 0. By definition, therefore, samples collected at day 0 had no protein-specific antibody titre.

Group and animal	Antigen	Days post-vaccination			Clinical outcome
		28	56	84	
Group A (2X CA/Tat-GST)					
CB139	CA	1:160	1:160	NT	Survived
	Tat	<1:5	<1:5	NT	
CB140	CA	1:20	1:80	NT	Died
	Tat	<1:5	<1:5	NT	
CB141	CA	1:20	1:40	NT	Survived
	Tat	<1:5	<1:5	NT	
CB142	CA	<1:5	1:10	NT	Survived
	Tat	<1:5	<1:5	NT	
CB143	CA	1:40	1:40	NT	Survived
	Tat	<1:5	<1:5	NT	
Group B (3X CA/Tat-GST)					
CB144	CA	1:80	1:160	1:160	Survived
	Tat	<1:5	<1:5	<1:5	
CB145	CA	1:640	1:640	1:640	Survived
	Tat	<1:5	<1:5	<1:5	
CB146	CA	<1:5	1:80	<1:5	Survived
	Tat	<1:5	<1:5	<1:5	
CB147	CA	1:160	1:320	1:640	Survived
	Tat	<1:5	<1:5	<1:5	
CB148	CA	1:40	1:40	1:80	Survived
	Tat	<1:5	<1:5	<1:5	
Group C (3X CA-GST + Tat-GST)					
CB149	CA	<1:5	<1:5	1:160	Survived
	Tat	<1:5	<1:5	<1:5	
CB150	CA	1:40	1:40	1:40	Survived
	Tat	<1:5	<1:5	<1:5	
CB151	CA	1:160	1:80	1:80	Survived
	Tat	<1:5	<1:5	<1:5	
CB152	CA	1:80	1:80	1:160	Died
	Tat	<1:5	<1:5	<1:5	
CB153	CA	<1:5	<1:5	1:40	Survived
	Tat	<1:5	<1:5	<1:5	

NT denotes not tested.

Response of vaccinates to infection with JDV_{Pul01}

Clinical outcome

All cattle inoculated with JDV_{Pul01} developed typical clinical signs of Jembrana disease, including swollen superficial lymph nodes, diarrhoea, loss of appetite and lethargy. One of the control cattle (CB156) died 13 days post-infection, one of the cattle vaccinated with the CA/Tat-GST polyprotein died 14 days post-infection (CB140; Group A) and one of the cattle inoculated 3 times with the individual CA-GST and Tat-GST proteins (CB152; Group C) died 14 days post-infection. A post-mortem examination was not conducted.

Febrile response after infection

An analysis of the febrile response in vaccinated and control groups is shown in Tables 5.7 and 5.8. All cattle inoculated with JDV_{Pul01} developed a febrile response 7-9 days after infection, and no differences in this incubation period were observed between any vaccinated group (Groups A, B and C) and the control group (Group D)

The maximum febrile temperature in Group A and Group C was significantly lower than in the control Group D ($p=0.01802$ and 0.027167, respectively) and when all vaccinated cattle (Groups A, B and C) were compared to the control group (Group D) vaccination significantly reduced maximum febrile temperatures ($p=0.00676$).

The fever score or area under the curve was lower in Group A than in the controls but not significantly different between cattle in Group B and the controls. The cattle that died (CB140, CB152 and CB156) had significantly lower fever scores ($p=0.023502$) than the cattle that survived and fever score did not, therefore, correlate with protection.

The duration of the febrile period categorized as the duration of low (39.3-40.2), moderate (40.3-41.2) or high (41.3+) fever or hyperpyrexia. All CA and Tat vaccinated groups had the same duration of low grade fever, the duration of moderate fever was longer in Group B than in the controls, and all vaccinated cattle had a significantly shorter period of high grade fever than the controls ($p=0.003843$).

Table 5.7. The febrile response to JDV infection following infection with JDV_{Pul01}, including the onset of the febrile response, the maximum temperature response, fever score (based on a standard area under the curve calculation), mean febrile temperature, the duration of fever and clinical outcome. Vaccinated cattle generally had lower mean febrile responses and significantly lower maximum febrile responses: only 4 vaccinated cattle out of 15 developed a rectal temperature of ≥ 41ºC whereas this occurred in 4 of the 5 control cattle.

Group and animal	Onset of fever after infection (days)	Max temperature	Fever score*	Mean febrile temp (ºC)	Duration of fever (h)**			Clinical outcome
					Low	Moderate	High	
Group A (2X CA/Tat-GST)								
CB139	8	40.7	3.5	40.375	24	72	0	Survived
CB140	8	40.6	2.9	40.225	48	48	0	Died day 14
CB141	7	41	4.1	40.32	96	48	0	Survived
CB142	7	40.5	2.6	40.15	96	24	0	Survived
CB143	8	40.8	2.9	40.225	96	48	0	Survived
Mean	7.6	40.72	3.2	40.259	72	48	0	1/5
Group B (3X CA/Tat-GST)								
CB144	7	41.2	6	40.5	72	96	0	Survived
CB145	7	41.2	6.6	40.44	72	96	0	Survived
CB146	7	40.9	4.7	40.44	72	48	0	Survived
CB147	8	40.8	4.9	40.31	72	96	0	Survived
CB148	7	40.6	3.9	40.28	48	72	0	Survived
Mean	7.2	40.94	5.22	40.394	67.2	81.6	0	0/5
Group C (3X CA-GST + Tat-GST)								
CB149	8	40.8	5	40.333	72	72	0	Survived
CB150	9	40.7	4.9	40.48	48	96	0	Survived
CB151	8	40.7	4.5	40.4	48	96	0	Survived
CB152	8	40.3	3.2	40.033	120	24	0	Died day 14
CB153	8	41.1	4.6	40.265	96	72	0	Survived
Mean	8.2	40.72	4.44	40.302	76.8	72	0	1/5
Group D (3X GST)								
CB154	8	41.1	5.6	40.62	48	96	0	Survived
CB155	7	41.3	5.1	40.58	96	48	24	Survived
CB156	8	41	3.1	40.12	72	48	0	Died day 13
CB157	9	40.8	2.6	40.15	96	24	0	Survived
CB158	9	41.8	4.7	40.44	48	48	24	Survived
Mean	8.2	41.2	4.28	40.382	72	52.8	9.6	1/5

*Fever score was calculated using area under the curve above 39.3°C

**Low fever 39.3-40.2ºC, moderate 40.3-41.2 ºC and high (hyperpyrexia) ≥ 41.3 ºC.

Table 5.8. Statistically significant differences in the febrile response between vaccinated and control groups after challenge with JDV_{Pul01}.

Parameter	Group		
	A 2 x CA/Tat-GST polyprotein	B 3 x CA/Tat–GST polyprotein	C 3 x CA-GST + Tat-GST proteins
Time from infection to onset of fever	NSD	Reduced (p=0.01)	NSD
Maximum febrile temperature	Reduced (p=0.02)	NSD	Reduced (p=0.01)
Mean febrile temperature	Significantly reduced 10-14 days post-infection	Increased 7-9 days post-infection	Significantly reduced 10 days and 13 days post-infection
Fever score	Reduced but not significantly (p=0.07)	NSD	NSD
Duration of fever (total)	NSD	NSD	NSD
Duration of fever (low)	NSD	NSD	NSD
Duration of fever (moderate)	NSD	Increased (p=0.05)	NSD
Duration of fever (high)	Reduced but not significantly (p=0.07)	Reduced but not significantly (p=0.07)	Reduced but not significantly (p=0.07)

NSD denotes no significant difference between vaccinated group and control group

Peripheral blood leucocyte changes

In response to infection there was a typical decrease in the numbers of circulating leucocytes was detected following infection with JDV_{Pul01} corresponding to the onset and duration of the febrile response but there were no detectable differences in mean leucocyte counts between the various cattle groups (Figure 5.5). However, the differential counts showed that the control group had less circulating lymphocytes and more granulocytes compared to the vaccinated groups of cattle (Figure 5.6 and 5.7). The lymphocyte numbers declined gradually in all cattle until day 11 post-infection when the lymphocyte numbers gradually rise again. All cattle developed a neutrophilia 3-9 days post-challenge and this was followed by a transient neutropenia lasting for 2-3 days before the numbers returned to their original levels. All cattle had high monocyte numbers from day 13 post-challenge onwards. There was little variation in eosinophil and basophil numbers due to their low numbers in blood except for the control group where increased granulocyte numbers were observed (Figure 5.6).

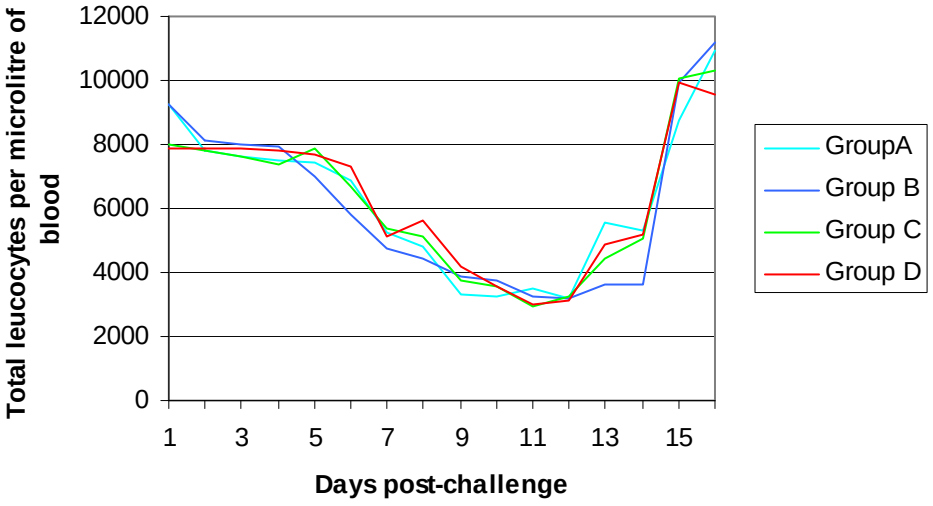
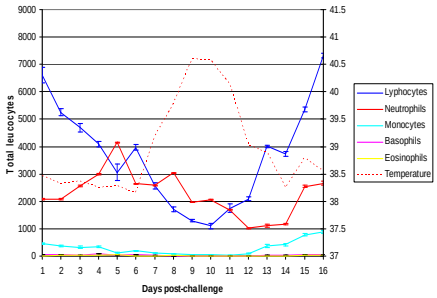
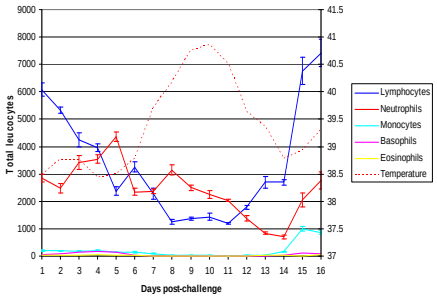


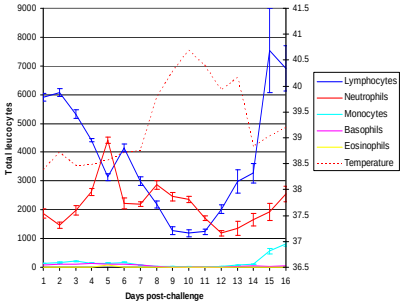
Figure 5.5. The mean total leucocyte counts of cattle in each of Groups A, B, C and D after infection with JDV_{Pul01} indicating only minor differences in total leucocyte levels between groups of cattle.

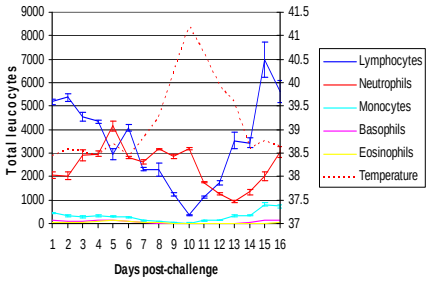
a b





c d





e

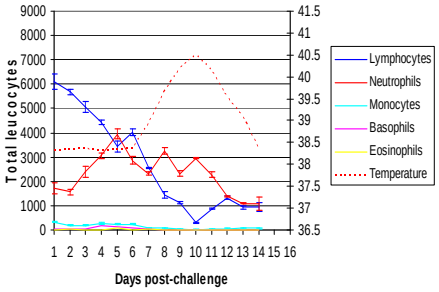


Figure 5.6. Comparison of leucocyte change shown as the group mean ± SE and mean rectal temperatures during the acute phase of infection in vaccinated and control groups. (a) Group A (JDV CA/Tat-GST vaccinated 2 times); (b) Group B (JDV CA/Tat-GST 3 times vaccinated); (c) Group C (JDV CA-GST + Tat-GST 3 times vaccinated); d, Group D (Control group, GST vaccinated 3 times); (e) 3 cattle that died during the trial. This shows that whilst the vaccinated cattle still developed lymphopenia it was not as severe as the control cattle and that the vaccinated cattle that died had a very similar profile to the control cattle indicating that these cattle failed to develop an adequate response to the vaccine by the day of challenge.

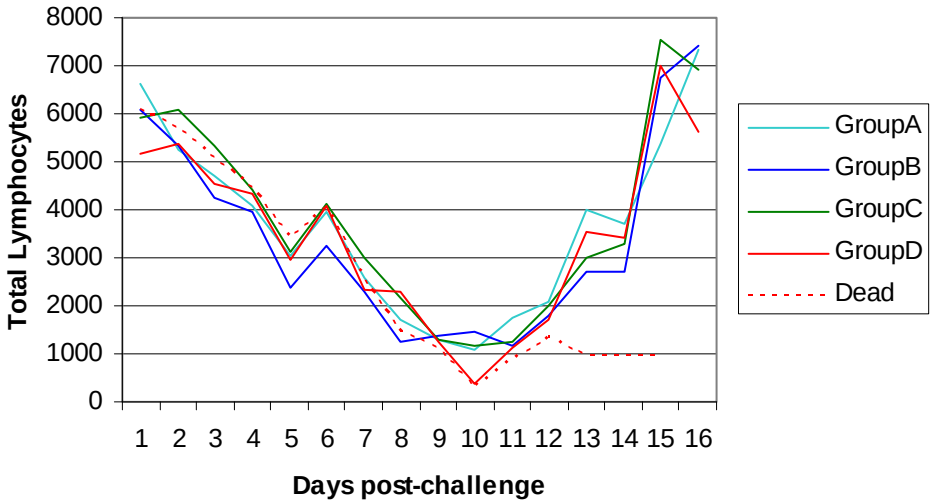
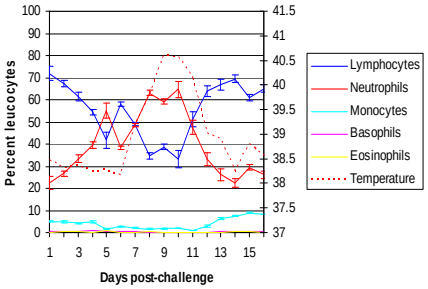


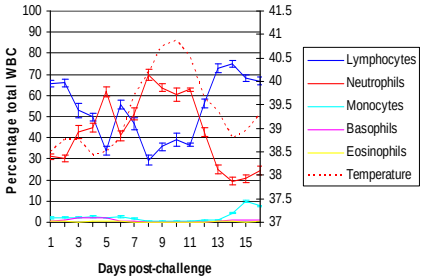
Figure 5.7. Mean lymphocyte counts in cattle in Groups A, B, C and D, and also in cattle that died during the course of the disease, following infection with $JDV_{PUL/01}$ showing that the vaccinated cattle developed significantly less severe lymphopenia (p=0.0025) during the acute phase of disease.

The vaccinated cattle all had less severe lymphopenia than the control cattle during the acute febrile period and did not develop the dramatic alteration in lymphocyte: neutrophil ratio typically seen in infection (Figure 5.8). The 2 vaccinated cattle that died had a very similar leucocyte dynamic response to the control group.

a



b



c

d

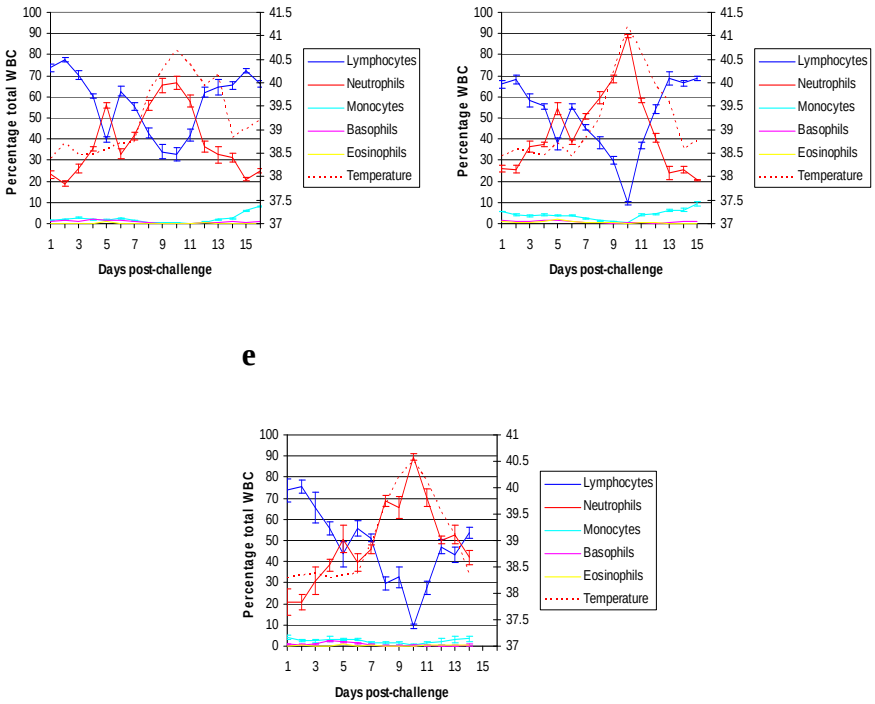


Figure 5.8. Graphs showing the mean percentage change in the leucocyte population in each group after infection with JDV_{Pul01}. (a) Group A (2x JDV CA/Tat-GST); (b) Group B (3x JDV CA/Tat-GST); (c) Group C (3x JDV CA-GST + Tat-GST); (d) Group D (Control group immunized with GST only); (e) 3 cattle that died (CB140, CB152 and CB156) during the course of the disease. The alterations in the leucocyte populations during the acute phase of infection were markedly less severe in the vaccinated cattle and the vaccinated cattle that died had a very similar profile to the control cattle.

There were a number of haematological observations during infection that have not been reported previously in JDV infected cattle. Seven of the 20 cattle had anisocytosis 8-10 days post-infection at the onset of the febrile response (Figure 5.9) and vacuoles were detected in the cytoplasm of lymphocytes and neutrophils 11-16 days post-infection (Figure 5.9). There was a marked regenerative left-shift seen in all 20 cattle 12-16 days post-infection with band neutrophils outnumbering neutrophils in the peripheral blood (Figure 5.9). A transient monocytosis was observed 14-15 days post-infection in 15 of the 17 cattle that survived.

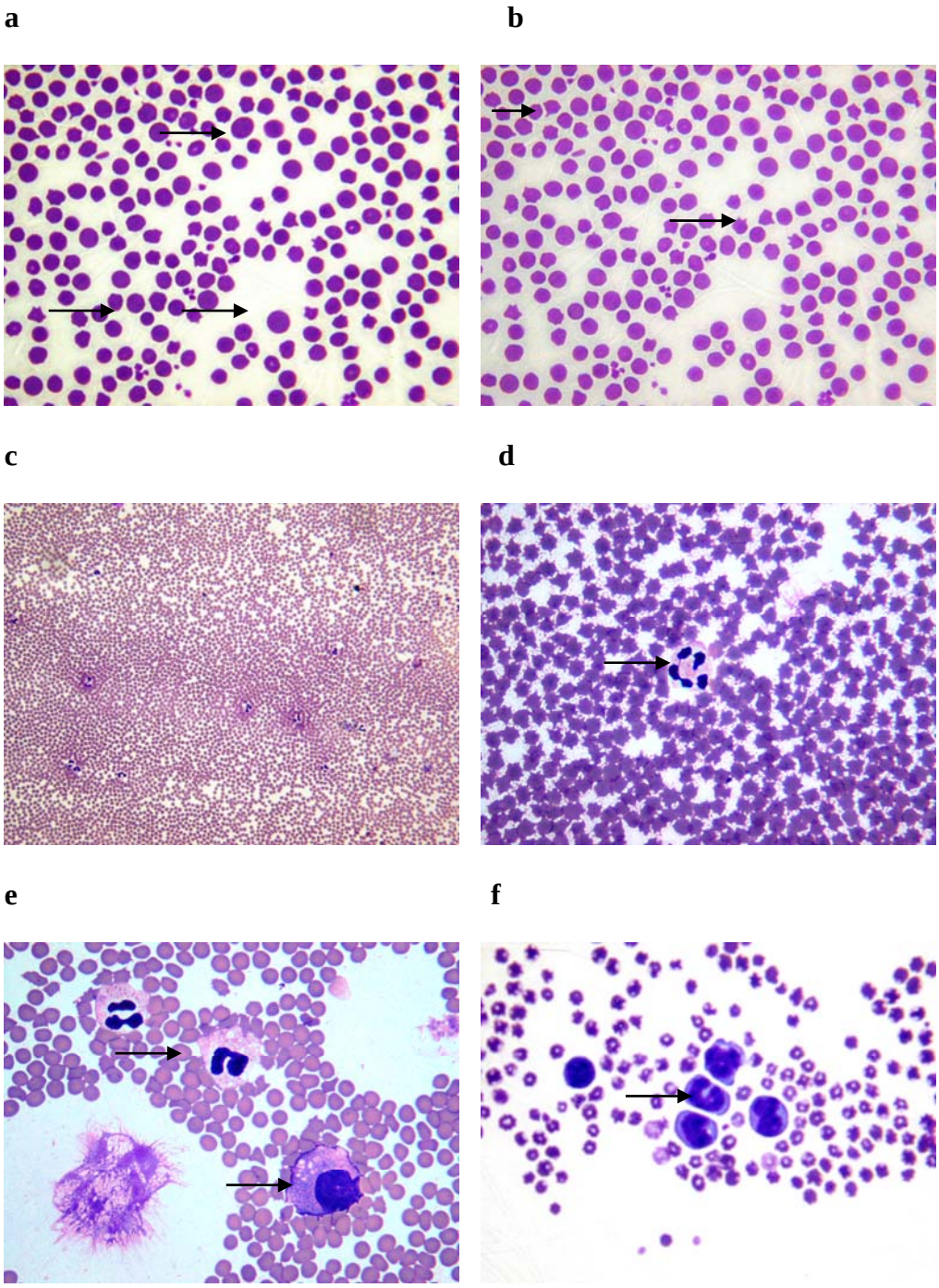


Figure 5.9. Abnormal bovine blood smears stained using the DiffQik (Difco) at 100X magnification: a, red blood cells showing marked anisocytosis; b, poikilocytosis (crenulated red blood cells); c, febrile period showing severe lymphopenia and neutrocytosis (20x magnification); d, neutrophil during the febrile period showing a segmented nucleus with grainy vacuolated cytoplasm.; e, Large activated lymphocyte showing grainy vacuolated cytoplasm with 2 neutrophils during the post-febrile period and f shows a regenerative left shift (band neutrophils outnumber mature neutrophils).

Table 5.8. Statistically significant differences in the WBC total numbers between vaccinated and control groups after challenge with JDV_{Pul01}.

Parameter	Group		
	A 2 x CA/Tat-GST polyprotein	B 3 x CA/Tat–GST polyprotein	C 3 x CA-GST + Tat-GST proteins
Leucocytes	Higher at days 1 and 11 Lower at days 9 and 10	Higher at days 1 and 11 Lower at days 5, 6 and 8	NSD
Lymphocytes	Higher at days 1, 10 and 11	Higher at day 10	Higher at day 10
Neutrophils	Lower at days 9 and 10	Lower at days 10 and 11	Lower at day 10
Monocytes	Lower at days 5, 10 and 11	Lower at days 1, 5, 11, 12, 13 and 14	Lower at days 1, 5, 11, 12, 13 and 14
Eosinophils	Lower at days 1, 4, 5, 6, 7 and 12	Lower at days 5 and 7	Lower at days 1, 4, 5, 6 and 11
Basophils	Lower at days 1, 3, 5, 8 and 11	Lower at days 8 and 11	Lower at day 7

NSD denotes no significant difference between vaccinated group and control group.

Plasma viral load

Vaccinated cattle had significantly higher viral load in plasma at days 3, 4 and 5 (p=0.021758, 0.036741 and 0.049547) and a higher but not significantly higher load at day 6 (p=0.061936). Two of the vaccinated cattle that died CB152 (Group C) and CB156 (GST control Group D) had developed very high viral loads at the time of death. The other animal that died (CB140, Group A) had a decreasing virus load at the time of death (Figures 5.10 and 5.11).

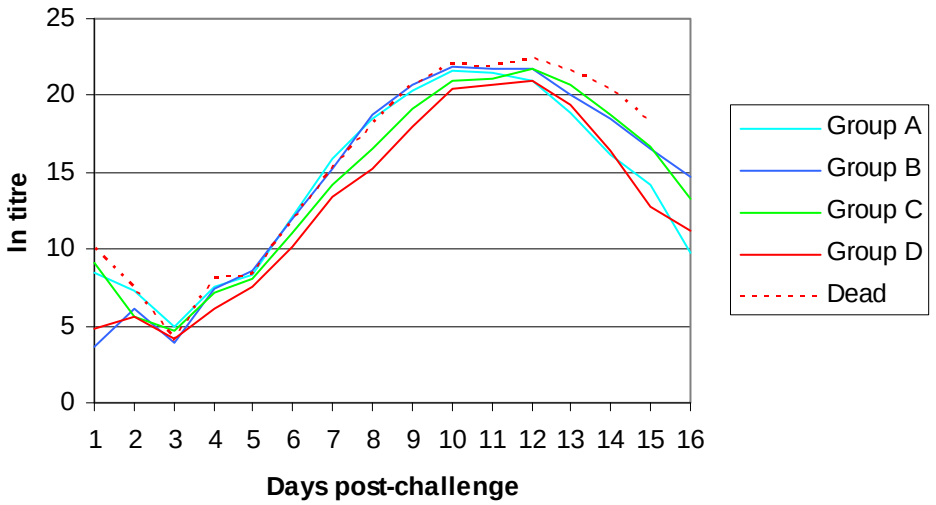
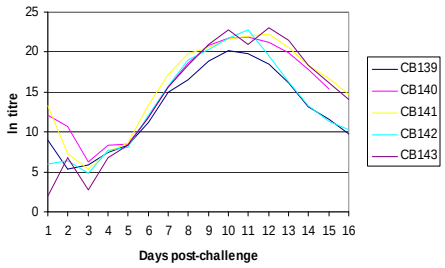
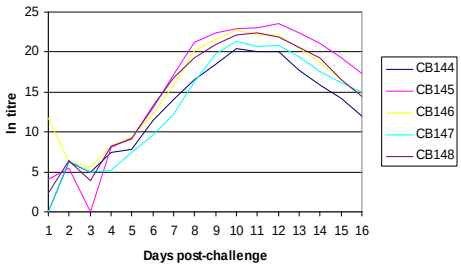


Figure 5.10. Graphs showing the mean change in viral titre/mL of plasma in each group after infection with JDV_{Pul01}. The graph depicts the mean plasma virus load during the course of the disease after infection of Group A (2x JDV CA/Tat-GST polyprotein), Group B (3x JDV CA/Tat-GST polyprotein), Group C (3x JDV CA-GST + Tat-GST), Group D (control group immunized with GST) and the 3 cattle that died (CB140, CB152 and CB156).

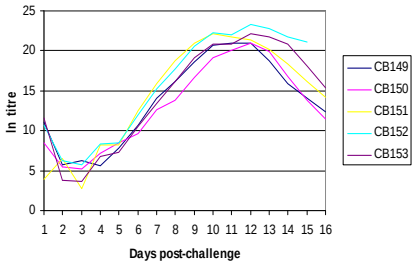
a



b



c



d

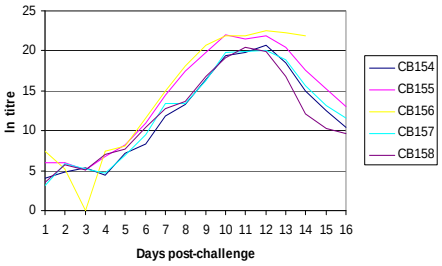


Figure 5.11. Natural log of viral load (genome copies/mL) determined by qRT-PCR in plasma of individual cattle in each group after infection with JDV_{Pul01}. (a) Group A; (b) Group B; (c) Group C; (d) Group D. Demonstrating the animal to animal variation between cattle in all groups.

Table 5.9. Statistically significant differences in the viral load between vaccinated and control groups after challenge with JDV_{Pul01}.

Parameter	Group		
	A	B	C
	2 x CA/Tat-GST polyprotein	3 x CA/Tat–GST polyprotein	3 x CA-GST + Tat-GST proteins
Viral load	Higher at days 4, 5, 7, 8 and 9	Higher at days 4, 5, 6 and 7	Higher at day 1

Discussion

An antibody response to the vaccine proteins was the only measurable indicator of the immunological status of the vaccinated cattle; assays for the detection of cell-mediated immunity were not considered feasible under the conditions available in Indonesia. The vaccinated cattle in all groups developed very high titre antibody responses to the JDV CA and Tat antigens, detected by both ELISA and Western immunoblot, and were very high at the time of infection with JDV. Cattle vaccinated with the fused JDV CA/Tat-GST developed higher titre vaccine specific responses than the cattle vaccinated with the individual CA-GST + Tat-GST.

Assuming appropriate immunising antigens are selected for use in a vaccine, a strong antibody response to the vaccine proteins can be a good indicator of vaccine efficacy and duration of protection (Letvin, 2002). However in lentiviruses this is not always correct and a strong antibody response to vaccination is not necessarily beneficial and immune activation can sometimes be associated with enhanced lentiviral replication (Fauci et al., 1991; Tirado and Yoon, 2003). Antibody-dependant enhancement of viral replication has been described with anti-Env and anti-Gag antibody responses in most lentiviruses (Hammond et al., 1999b; Hosie et al., 1992; Nenci et al., 2007; Tirado and Yoon, 2003; Villinger et al., 2003) but it has not been observed in cattle vaccinated with the tissue-derived and inactivated whole virus JDV vaccine which has elicited a strong response to several viral proteins (Hartaningsih et al.,2001).

There was a strong IgG, IgA and IgM antibody response to the JDV CA antigen, which should be advantageous in the provision of a protective immunity. An especially strong IgG response to CA and Tat developed in all cattle after vaccination but the cattle that died appeared to not have a lesser IgG response to the vaccine proteins. It was noted that the cattle that appeared to develop the strongest protective immunity were those vaccinated only twice with the CA/Tat-GST polyprotein and it is possible that this was due to the reduced dose (total 8 mg instead of 12 mg when 3 vaccinations were administered) with less stimulation of the immune system. The IgG antibody is the main serum antibody and consists of IgG1, IgG2, IgG3 and IgG4 sub-classes (Butler, 1983) that are involved in secondary immune responses (antigen recognition). For this study a generic anti-bovine IgG antibody was used which was unable to differentiate between the sub-classes such as IgG1 and IgG3 responses which have been shown to take longer to develop than the other IgG subclasses in

horses and this may be important to vaccine efficacy (Hammond et al., 1997). Further investigation into the time necessary to develop a mature humoral immune response and the vaccine dose needed to elicit protection should be undertaken as some cattle vaccines can take up to 21 weeks to elicit a mature protective humoral immune response (Wedlock et al., 2005).

All the vaccinated cattle except for 2 (CB146 in Group B and CB153 in Group C) also developed a detectable CA-specific IgM response by the day of challenge but only one animal (CB147 in Group B) had developed a Tat-specific IgM response (this animal also had a high CA IgM titre) at challenge. IgM has low affinity but high avidity allowing the IgM to recognize a large variety of novel pathogens during the primary immune response. IgM is also a complement-activator and can stimulate antigen-specific IgG production necessary for immunity to pathogens (Boes, 2000).

All of the vaccinated cattle also developed an IgA response to the CA antigen by the day of virus infection 28 days after the last vaccine dose. Tat-specific IgA was not detected against the Tat antigen but it may take longer for the IgA response to mature to the less immunogenic Tat. IgA is an important part of the body's mucosal immunity but is also a potent neutralising antibody that can act on the epithelial surface or more importantly within the infected epithelial cells to prevent viral replication and allow effective viral clearance (Mazanec et al., 1993). An IgA response is therefore important due to its neutralising activity and its possible role in preventing infection through mucosal surfaces (Bomsel et al., 1998; Butler, 1983; Mazanec et al., 1992; Roitt and Delves, 2002). One study found that HIV Env specific IgA transcytosed through the epithelial cell wall and efficiently neutralized HIV replication in the epithelial cells (Huang et al., 2005). It would be interesting to determine if in "atypical" responders to JDV infection (Ditcham, 2007) control replication via serum IgA-mediated neutralisation of virus similar to that seen in HIV-2 non-progressors (Lizeng et al., 2004).

While the strong antibody response to the polyprotein vaccine suggests it was highly immunogenic, the indications of the induction of a protective efficacy were less clear. There was, however, evidence of an amelioration of the disease process after infection in most vaccinated animals, especially regarding the febrile response to infection. Vaccinated cattle generally had longer febrile responses than control cattle but had significantly lower maximum febrile temperature and none developed hyperpyrexia

which is often associated with the disease. This was particularly evident in those cattle vaccinated twice with the CA/Tat polyprotein (Group A). These cattle had reduced fever score, a reduced mean febrile temperature, a reduced maximum febrile temperature, and a reduced period of high fever (hyperpyrexia) compared to the controls. High grade fevers are often associated with increased pathogenesis and mortality (Blatteis, 2007; Blatteis and Sehic, 1998). The results obtained in the group vaccinated twice with the CA/Tat polyprotein were at least equivalent to those observed in the cattle vaccinated 3 times with the mixture of CA-GST and Tat-GST proteins (Group C) and surprisingly, better than those observed in the cattle vaccinated 3 times with the CA/Tat polyprotein. The cattle vaccinated 3 times with CA/Tat-GST had a reduced incubation period from infection until the onset of the febrile response, a higher mean fever than the controls 7-9 days post-infection, and an increased period of moderate fever compared to the controls.

The 2 vaccinated cattle that succumbed to infection and died 14 days post-challenge failed to respond to vaccination in the manner seen by the other 13 vaccinated cattle and had very similar clinical signs and haematological changes to the control cattle with high febrile responses and severe lymphopenia. The duration of the period from the first day of fever to the peak of fever was significantly shorter in these cattle than in cattle that survived. The cattle that died (CB140, CB152 and CB156) had significantly lower fever scores than the cattle that survived indicating that this parameter did not correlate with protection and may therefore be useful for determining disease outcome.

The vaccinated cattle had significantly higher levels of circulating lymphocytes and a disturbance of the typical lymphocyte: neutrophil ratio (1:0.5) by the day of challenge indicating that the cattle were still mounting a humoral immune response to the vaccine proteins at the time of virus infection. This is further supported by the ELISA results prior to challenge that show the titre of anti-CA IgG, IgM and IgA was still increasing on the day of challenge. The anti-Tat IgG response was also increasing on the day of challenge. This immune stimulation at the day of challenge is undesirable in vaccine trials because the immune response to the vaccine has not fully matured and vaccine efficacy cannot be evaluated accurately (Cole et al., 1998; Hammond et al., 1999b; Montelaro et al., 1998). It has been reported that an immature immune response can also be responsible for enhancement of infection or complete lack of

vaccine protection (Matteucci et al., 1999; Montelaro et al., 1998) and ideally a greater period of time should be left after the final vaccine dose before challenge to generate valid vaccine efficacy data, although this obviously adds to the cost of these already expensive trials as reported previously (Ditcham, 2007; Soeharsono et al., 1995a)

The level of leucopenia in the vaccinated and control animals did not appear to differ significantly but there was less reduction of lymphocytes in the vaccinated groups compared with the control group during the febrile period, although there was no significant difference between any of the vaccinated groups. The vaccinated cattle had significantly greater minimum lymphocyte numbers and significantly less days of severe lymphopenia (<1,000 lymphocytes/μL of blood) suggesting that immunisation with the CA and Tat proteins induced a protective immune response in the majority of vaccinated cattle. Not surprisingly 3 of the 4 cattle with the lowest total number and percentage of lymphocytes were the cattle that died.

The analysis of the leucocyte subpopulations yielded additional information about the effects of the disease that has not been previously reported. Soeharsono *et al* 2001 reported a mild neutropenia associated with experimental JDV infection and while a mild neutropenia after the febrile period was observed in these cattle there was in fact a neutrocytosis or neutrophilia during the acute febrile period where up to 90% of the total circulating leucocytes were neutrophils. This up-regulation of circulating neutrophils is physiologically important because their half life is generally between 4 to 10 h when not activated and once activated they rupture causing inflammation so all the daily changes in neutrophil numbers are due to up-regulation of neutrophils by JDV infection (Roitt and Delves, 2002). A similar pattern of up-regulation of neutrophils has been observed in HIV-1 infection where studies have shown that neutrophils bind the virions and transfer infection to activated lymphocytes more efficiently than HIV virions alone (Gabali et al., 2004). These changes may suggest that the neutrophilia may be associated with the spread of the virus to naïve tissues throughout the infected cattle 3 days before the peak of the febrile responses. A further study determined that the neutrophilic burst of reactive oxygen intermediates (ROI) strongly activated HIV-1 long terminal repeats (LTR) indicating that this may be an important mechanism of acute viral activation (Klebanoff and Headley, 1999). The decline in neutrophil levels from day 9-14 in all groups leading to the mild

neutropenia confirms previous studies and has also been reported in FIV infections (Hofmann-Lehmann et al., 1995; Soeharsono et al., 1995a; Soesanto et al., 1990). There was a strong correlation between the percentage of neutrophils in circulation and the rectal temperatures suggesting that the neutrophils were a significant cause of the inflammatory response during the acute febrile period. Coinciding with the peak in absolute and percentage change in neutrophils at 5 days post-challenge, there was also a rise in absolute numbers of the other granulocytes (basophils and eosinophils). This is similar to what has been found 2-4 weeks post-challenge with FIV, which suggested a Th2 response to components of the live virus challenge (Hofmann-Lehmann et al., 1995).

In this current experiment, vaccination with CA and Tat did not effect any reduction of the plasma virus load, in contrast to results reported previously (Ditcham, 2007). The CA/Tat-GST vaccinated cattle in this current experiment developed higher viral titres during infection particularly in the early phase of the disease (days 3, 4, 5 and 6) which may be due to the cattle still responding to the vaccine by the day of challenge and having higher circulating lymphocytes for the virus to infect. This conclusion is supported by the fact that the cattle with the highest circulating lymphocytes also had increased early viral replication and the cattle in group C which did not have significantly higher circulating lymphocytes by the day of challenge also did not have significantly higher early viral replication. Interestingly the increase in viral load in the vaccinated cattle did not necessarily increase clinical signs or severity of disease. This suggests that vaccination allows the cattle to respond better to higher titres of virus without the hyperpyrexia and severe lymphopenia usually associated with the pathogenesis.

There may have been factors that reduced the efficacy of the recombinant CA/Tat-GST polyprotein vaccine that need to be addressed in future experiments. The procedures used in attempts to induce a protective immune response in the current experiment were in many cases based on arbitrary decisions and may not have been correct. Improved results could have been obtained with different methodology. The design of any vaccine trial must consider the dose, route of administration, time between vaccinations, time between final vaccination and challenge, identification of correlates of protection, whether to use homologous or heterologous challenge, route of challenge and challenge dose, all of which can affect the outcome (Dunham, 2006).

Testing of all of these parameters was not economically feasible and the current experiment was a continuation of a series of vaccine trials undertaken with limited resources and time.

The quantity of recombinant protein in each vaccine dose in the current experiment was likely to have been excessive. Preliminary work conducted by others had shown that 3 vaccinations with doses of 2 mg of JDV CA-GST and 2 mg of Tat-GST reduced viral load and ameliorated the disease after challenge (Ditcham, 2007). The dosage of 4 mg of he JDV CA/Tat-GST polyprotein in the current experiments was extremely large, compared to what has been used with other experimental lentivirus vaccination attempts. Horses have been vaccinated with between 50-200 μg per dose of recombinant EIAV Env protein, similar urea/DTT solubilised recombinant protein vaccines for sheep contained 50 μg per dose, and other recombinant protein cattle vaccines contain between 50 -200 μg per dose (Hammond et al., 1999a; Issel et al., 1992; Purcell et al., 2003; Rothel et al., 1997; Shokri and Jafarzadeh, 2001; Wu et al., 2004). Earlier recombinant protein vaccines tested had low purity, contained little viral antigen per dose, whereas the JDV CA/Tat-GST polyprotein had much higher purity than the earlier recombinant protein vaccines that were tested and the large dosage used in the current experiment could have been detrimental to vaccine efficacy (Fife et al., 2004). The next stage of vaccine development should include a dose/response trial with the polyprotein vaccine to determine the optimum dose that will maintain and perhaps increase efficacy and decrease the final cost of production.

There was evidence that the immune response at the time of infection had not matured and was still developing, and that a longer time interval after infection should have been used. In the current experiment, the period between vaccinations was 28 days with either 2 or 3 vaccinations and viral challenge 28 days after the final vaccination. The short intervals of 28 days were chosen to reduce the total period required to house the cattle, to reduce costs and reduce stress for the animals in the tropical environment where the experiments were conducted. The short interval between vaccinations failed to take into account cell-mediated responses which can take up to 8 months post-vaccination to develop (Fanales-Belasio et al., 2002; Hosie and Flynn, 1996). Two of the vaccinated cattle (CB140 and 152) be protected by vaccination and this was probably due to insufficient time for the vaccine-specific responses to mature and the humoral response to the vaccine was probably still developing when the cattle were

challenged. Further studies should take into account the time needed for maturation of the humoral immune response and cell-mediated response to the viral antigens and compare efficacy of the different vaccine regimes especially in light of new research that the HIV-1 Tat protein broadens T-cell responses against the Gag and Env viral proteins (Gavioli et al., 2008; Gavioli et al., 2004). Maybe responses to T cell epitopes on the CA antigen important for protection against Jembrana disease may need more time to mature between vaccination and challenge. The challenge dose of 1,000 ID_{50} of the $JDV_{PUL/01}$ strain was previously found to reliably induce severe Jembrana disease in cattle (Ditcham, 2007; Stewart et al., 2005) and this is why it was used in these experiments but a challenge dose of 1,000 ID_{50} is considered extremely high in lentivirus challenge trials and most groups have attempted to use a low challenge dose to mimic naturally infections and this may have further masked vaccine efficacy (Finerty et al., 2000; Horton et al., 2002; Hosie and Flynn, 1996). The tissue-derived inactivated whole virus vaccine was tested for efficacy using 100-400 ID_{50} of a less pathogenic homologous JDV_{Tab87} strain (Hartaningsih et al., 2001). It would have been preferable to use a lower standard and quantified challenge dose of JDV that could have been stored in liquid nitrogen and used repeatedly, to overcome experiment to experiment variation. However, supplies of liquid nitrogen in Indonesia are unreliable and this has led to the use of donor animals to provide the infectious inoculum, which are infected with frozen (-80ºC) spleen tissue in which the infectious virus seems to survive for prolonged periods (Soeharsono et al., 1990).

Chapter 6. The response to and safety of a recombinant CA/Tat-GST polyprotein vaccine in cattle

Summary

A bacterially expressed recombinant JDV CA/Tat-GST polyprotein (Chapter 4) emulsified in a mineral oil adjuvant and previously tested in small scale laboratory trials as a potential vaccine for the control of Jembrana disease in Indonesia (Chapter 5) was further tested for safety and immunogenicity in a field trial in Bali. One hundred cattle were inoculated twice with the polyprotein vaccine and a similar number of cattle were inoculated with a control vaccine containing chicken ovalbumin and recombinant GST in a mineral oil adjuvant. Strong antibody responses to the CA and Tat proteins were detected for 12 months post-vaccination. A low prevalence of adverse reactions to CA/Tat-GST polyprotein vaccine occurred, including abortion and shock, but was also observed in the control group vaccinated with GST plus chicken ovalbumin only.

Introduction

In Indonesia, attempts are being made to control the spread of Jembrana disease by restricting the movement of cattle from endemic areas to Jembrana disease-free areas and ring vaccinating around new outbreaks in endemic areas with a tissue-derived inactivated whole virus vaccine (Hartaningsih et al., 2001). The preparation of this whole virus vaccine from spleen tissue requires the use of donor animals obtained from Nusa Penida, an island adjacent to Bali where the disease does not occur; the vaccine is expensive and its use is associated with production difficulties, safety and ethical issues that make it commercially unviable.

A variety of JDV recombinant proteins have been tested during small scale laboratory trials for efficacy as potential vaccines against Jembrana disease (Ditcham, 2007). A combination of 2 separate GST-tagged recombinant JDV CA and Tat proteins

expressed in BL21 *E. coli* using a pGEX expression system provided the best level of protection, in some experiments equivalent to those obtained using the tissue-derived inactivated whole virus vaccine.

As the use of 2 separate proteins was thought to make the large scale production of a vaccine utilising these proteins too expensive and difficult in Indonesia, a single plasmid construct expressing both CA and Tat proteins as a GST-tagged polyprotein was developed (Chapter 4) and the expressed polyprotein was tested for efficacy in small scale laboratory experiments (Chapter 5). In this Chapter, results of a further larger scale field trail of this polyprotein vaccine are reported. The vaccine was inoculated twice one month apart into 100 cattle and an additional 100 cattle were inoculated with a control vaccine containing recombinant GST and chicken ovalbumin. All inoculated cattle were maintained under field conditions by the owners and were monitored for 12 months to assess the clinical and immune response to the vaccine components.

Materials and methods

Vaccine preparations

A CA/Tat-GST recombinant polyprotein was produced as described in Chapter 4. The assessment of the polyprotein for purity and the presence of contaminating endotoxins and cell and plasmid-derived nucleic contamination was performed as described in Chapter 4. The preparation of recombinant GST for inclusion in the control vaccine was as described in Chapter 5. The JDV CA/Tat-GST recombinant protein vaccine that was produced was 95% pure by densitometry with a concentration of 6.48 mg/mL, providing a final yield of 0.68 g/l of culture (Figure 6.1). The recombinant GST protein produced had purity greater than 95% by densitometry with a concentration of 4.87 mg/mL, providing a final yield of 0.13 g/L (Figure 6.2). The final CA/Tat-GST polyprotein used for vaccination contained 4 endotoxin units/mg of protein.

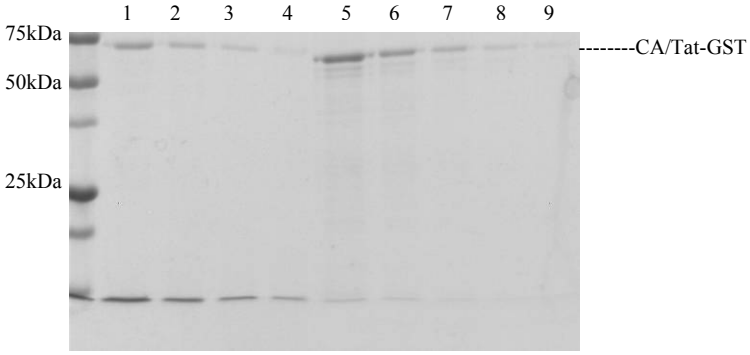


Figure 6.1. A Coomassie Brilliant Blue stained SDS-PAGE gel used to determine purity and concentration of the JDV CA/Tat-GST polyprotein incorporated into the vaccine. Lane 1, 1,000 ng of BSA and lysozyme; lane 2, 500 ng of BSA and lysozyme; lane 3, 250 ng of BSA and lysozyme; lane 4, 125 ng of BSA and lysozyme; lane 5, 1 µl JDV CA/Tat-GST; lane 6, 0.5 µl JDV CA/Tat-GST; lane 7, 0.25 µl JDV CA/Tat-GST; lane 8, 0.1 µl JDV CA/Tat-GST; lane 9, 0.05 µl JDV CA/Tat-GST. This SDS-PAGE gel shows the final purity and concentration of the CA/Tat-GST vaccine used for the safety trial.

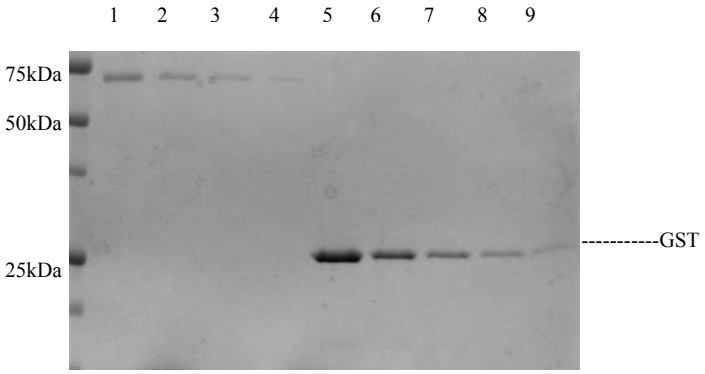


Figure 6.2. Coomassie Brilliant Blue stained SDS-PAGE gels to determine purity and concentration of the recombinant GST protein preparation incorporated into the control vaccine. Lane 1, 1,000 ng of BSA; lane 2, 500 ng of BSA; lane 3, 250 ng of BSA; lane 4, 125 ng of BSA; lane 5, 4 µl JDV recombinant GST; lane 6, 2 µl recombinant GST; lane 7, 1 µl recombinant GST; lane 8, 0.5 µl recombinant GST; lane 9, 0.1 µl recombinant GST. This SDS-PAGE gel shows the final purity and concentration of the recombinant GST used for the safety trial.

Chicken ovalbumin (Sigma) was prepared as a 1 mg/mL solution in solubilisation buffer and stored frozen at -20°C until required.

The final vaccine preparations administered to cattle were prepared essentially as described in Chapter 5 but a mineral oil adjuvant (MOA) supplied by Vaksindo was utilized instead of incomplete Freund's adjuvant (IFA). Each dose of the JDV

CA/Tat-GST polyprotein vaccine consisted of 2 mL (2 mg/mL) protein solution emulsified with 2 mL of mineral oil adjuvant. The control preparation contained 1 mL of chicken ovalbumin (1 mg/mL) and 1 mL of recombinant GST (1 mg/mL) emulsified with 2 mL of mineral oil adjuvant. The vaccine was administered intramuscularly in a single injection site on the side of the neck.

Vaccine trial

Two hundred Bali cattle were made available for this study from 2 neighbouring regions of the Tabanan district of Bali, an area where Jembrana disease is recognized as endemic. The cattle were mostly female of various ages and pregnancy status and had not been vaccinated previously with the tissue-derived whole virus vaccine. The cattle were owned by local smallholder farmers who had agreed to participate in this study under the direction of the provincial veterinary service. At the time of vaccination, a multivitamin complex was also injected in a separate site as an incentive for farmer participation in the vaccine trial.

The age distribution amongst the cattle utilized for the trial ranged from <23 months to >120 months old as shown in Table 6.1. Of the 200 cattle, 36.5% were female and pregnant, 58.5% were female and not pregnant, 4% were female but with an undefined pregnancy status, and 1% were male (Table 6.2).

Table 6.1. The age distribution of 200 cattle used in the safety trial. The control group were vaccinated with 1 mg of GST + 1 mg of chicken ovalbumin and the vaccinated cattle received 4 mg of CA/Tat-GST polyprotein. All cattle were vaccinated twice, 28 days apart.

Age (months)	Control group	Vaccinated group
0-23	13	15
24-47	22	26
48-71	35	23
72-95	23	15
96-119	3	14
≥120	4	7

Table 6.2. The pregnancy status of the 200 cattle before vaccination with either 1 mg of GST plus 1 mg chicken ovalbumin (control group) or 4 mg of CA/Tat-GST polyprotein (vaccinated group).

Status	Control group	Vaccinated group	Total
Male	0	2	2
Not pregnant	60	57	117
Unsure	5	3	8
1 month pregnant	4	0	4
2 months pregnant	5	1	6
3 months pregnant	6	9	15
4 months pregnant	6	5	11
5 months pregnant	0	5	5
6 months pregnant	4	3	7
7 months pregnant	5	4	9
8 months pregnant	4	10	14
9 months pregnant	1	1	2

All participating farmers were questioned on the previous health status of their cattle and were further questioned regarding the continuing health status of the cattle on each occasion when samples were obtained. None of the cattle were suspected by the owners to have had Jembrana disease in the immediate months preceding the trial.

Serological response to vaccination

Sera were collected from the 200 cattle in the 2 groups at day 0 (day of initial vaccination), week 4 (second vaccination), week 8, week 14, week 30 and week 52 after the initial vaccine dose.

All sera were tested for antibody to the whole virus antigen by Western immunoblot and an ELISA was used to quantify the CA and Tat protein-specific IgG, IgM and IgA as previously described in Chapter 5. The JDV Tat protein used as an antigen was expressed as a biotinylated protein with the PinPoint system and the histidine-tagged JDV CA protein was expressed with the pTrcHis system, as described in Chapter 5. All data was analysed using a Students 2 sample t-test to identify significant differences.

Comparison of adjuvants

As IFA was considered too expensive for large scale vaccine program in Indonesia, MOA was used instead of IFA in this trial. To compare the efficacy of the adjuvants to raise a humoral immune response to both the CA and the Tat portion of the vaccine, 5 seronegative cattle from the trial (B15-19) were compared with 5 cattle vaccinated with the CA/Tat-GST (IFA) and previously reported in Chapter 5. All data were analysed using a Students 2 sample t-test to identify significant differences in antibody titre to vaccination.

Results

Serological status of cattle before vaccination

Prior to vaccination, the prevalence of antibody to JDV CA protein in the control and CA/Tat-GST vaccinated group was 17% and 6%, respectively, determined by whole virus Western immunoblotting.

The age distribution of the antibody-positive cattle in the 2 groups is shown in Figure 6.3. None of the 76 cattle < 47 months of age were seropositive to JDV CA or Tat whilst the percentage of seropositive older cattle increased with age. There was a strong positive trend between age and seroprevalence (R^2 value=0.9901).

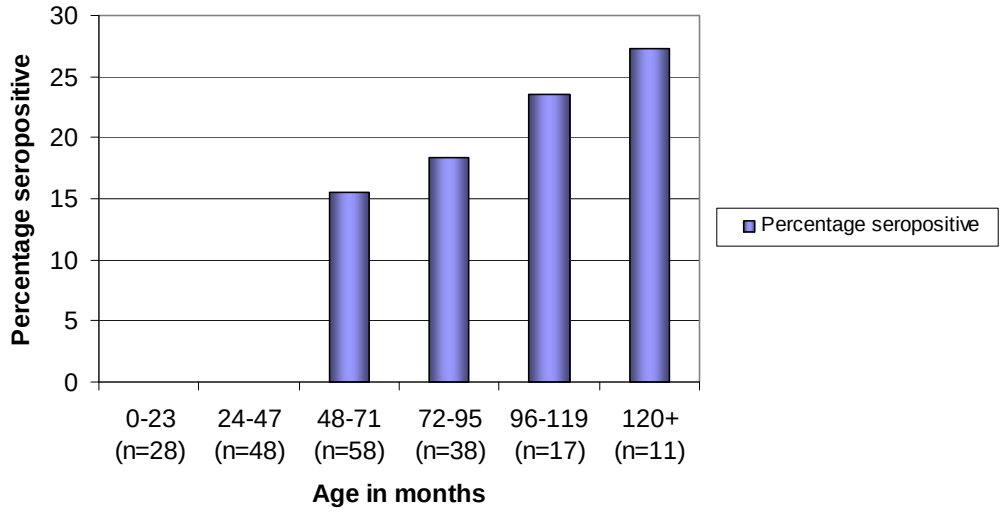


Figure 6.3. A demographic analysis of the age versus JDV antibody status of the 200 cattle from the Tabanan region of Bali prior to vaccination, demonstrating that no cattle < 4 years of age were seropositive and the proportion of seropositive animals increased with the age of the cattle.

Adverse reactions to vaccination

In the vaccinated group, abortion was reported. The first abortion occurred 20 days after the first vaccine dose in a 3 month pregnant heifer and the second and third abortion occurred within 4 days of the second vaccine dose in 5 and 8 month pregnant heifers. An anaphylactic-like (shock) reaction was observed in 2 of the vaccinated group when clinical signs of a rapid onset of dyspnea, hypersalivation and collapse occurred. One heifer in the vaccinated group had such a reaction about 5 min after the first vaccine dose and was doused with cold water and recovered. A second heifer had such a reaction about 7 min after the second vaccine dose and was immediately injected with anti-histamine (Deladril®) and recovered.

In the control group, 2 cattle aborted within 7 days of the second control vaccine dose. One heifer developed an anaphylactic-like reaction 5 min after the first vaccine dose and was treated with anti-histamine (Deladril®) and recovered (Table 6.3). There were some local reactions including swelling and sensitivity to touch at the injection site observed after vaccination in the vaccinated and control groups but there was no recorded data on the overall numbers in the trial.

Table 6.3. Abortion and anaphylactic-like (shock) reactions detected in vaccinated and control groups after 2 vaccinations.

Status	Control group		Vaccinated group		Total	
	Abortion	Shock	Abortion	Shock	Abortion	Shock
Male	0	0	0	0	0	0
Not pregnant	0	1/60	0	2/57	0	3
Undetermined	0	0	0	0	0	0
1 month pregnant	0	0	0	0	0	0
2 months pregnant	0	0	0	0	0	0
3 months pregnant	0	0	1/9	0	1	0
4 months pregnant	1/6	0	0	0	1	0
5 months pregnant	0	0	1/5	0	1	0
6 months pregnant	0	0	0	0	0	0
7 months pregnant	0	0	0	0	0	0
8 months pregnant	1/4	0	1/10	0	2	0
9 months pregnant	0	0	0	0	0	0

Serological response to CA after vaccination

In the vaccinated group, 6 cattle were seropositive to the CA protein prior to vaccination. One month after the first vaccine dose, 98 were positive by Western immunoblot and ELISA and by 2 months after the initial vaccine dose all 100 had seroconverted and remained positive until the end of the trial (Table 6.4). The titre of the CA response peaked 3 months after the initial vaccine dose, declined between 6 and 12 months after the initial vaccine dose but remained at a high level until 12 months after the initial vaccine dose when the observations were discontinued (Figure 6.4).

Table 6.4. Number of cattle that were seropositive or became seropositiveafter vaccination to JDV CA antigen at 0, 1, 3, 6 and 12 months after the initial vaccination.

	Months after initial vaccine dose				
	0	1	3	6	12
CA/Tat-GST vaccinated	6/100	98/100	100/100	100/100	100/100
Control	17/100	17/98	17/95	15/84	7/39

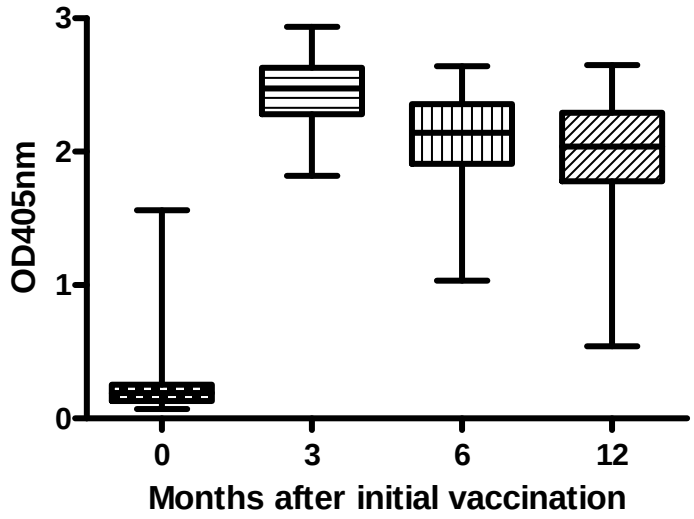


Figure 6.4. Boxplot analysis of the JDV CA antibody response showing the mean OD_{405} of the ELISA response at intervals after the initial vaccine dose. The upper and lower edges of the boxes correspond to the 75th and 25th percentiles, respectively, the central line represents the median and the vertical lines the range of values.

Serological response to Tat after vaccination

In the vaccinated group, 2 cattle were seropositive to the Tat antigen on the day of the initial vaccination and 98 cattle were seropositive 1 month after the initial vaccine doses when the second vaccine dose was administered. By 3 months after the initial vaccination all 100 cattle were positive to the Tat antigen and remained positive until 12 months after the initial vaccination (Table 6.5). The anti-Tat response peaked 3 months post-vaccination and markedly declined at 6 months but was then maintained until 12 months after the initial vaccine dose (Figure 6.5).

Table 6.5. The total numbers of cattle that were seropositive to the JDV Tat antigen at 1, 3, 6 and 12 months after the initial vaccine dose.

	Months after initial vaccine dose				
	0	1	3	6	12
CA/Tat-GST vaccinated	2/100	98/100	100/100	100/100	100/100

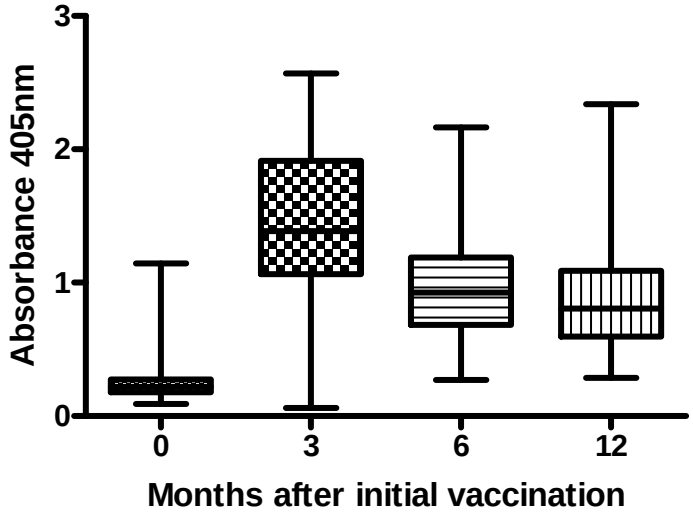


Figure 6.5. Boxplot analysis of the JDV Tat antibody response showing the mean OD_{405} of the ELISA at intervals after the initial vaccination. The upper and lower edges of the boxes correspond to the 75th and 25th percentiles, respectively, the central line represents the median and the vertical lines the range of values.

Comparison of vaccine specific titres to JDV CA and Tat antigens with different adjuvants

The antibody titres obtained in this safety trial were compared to those reported previously in Chapter 5 in 5 cattle vaccinated twice with the same JDV CA/Tat-GST polyprotein but where IFA was used instead of MOA used in the field trial. The 5 cattle inoculated with the CA/Tat-GST polyprotein emulsified in IFA developed significantly higher IgG titres to the CA antigen at 1 month ($p=0.032$) and 2 months after the initial vaccination ($p=0.004$). The cattle vaccinated with the CA/Tat-GST

polyprotein vaccine emulsified in IFA also developed significantly higher IgG titres to the Tat antigen 1 month after the initial vaccination and higher but not significantly higher (p=0.052) titres 2 months after the initial vaccination (Figure 6.6 and 6.7).

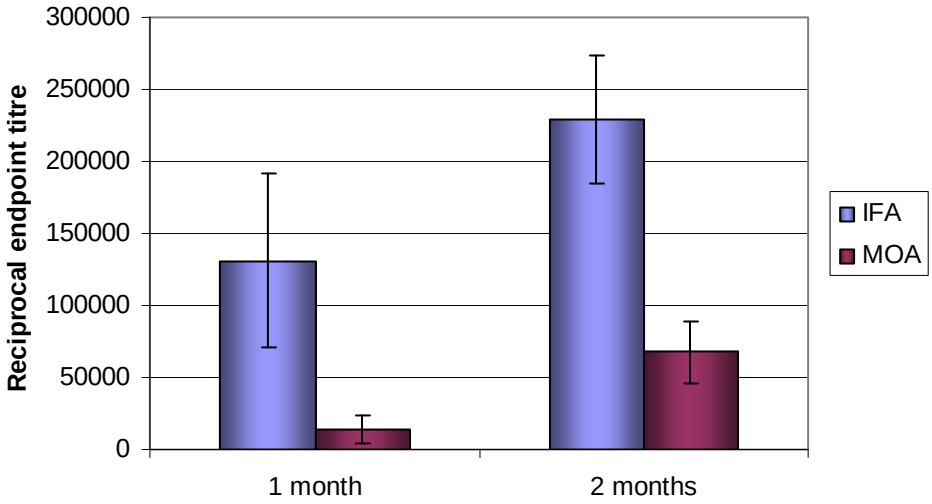


Figure 6.6. Graphical representation of the IgG anti-CA titres obtained with the IFA (results from Chapter 5) and MOA adjuvants (results from this Chapter) showing that the use of IFA adjuvant resulted in a much greater humoral immune response to the CA protein.

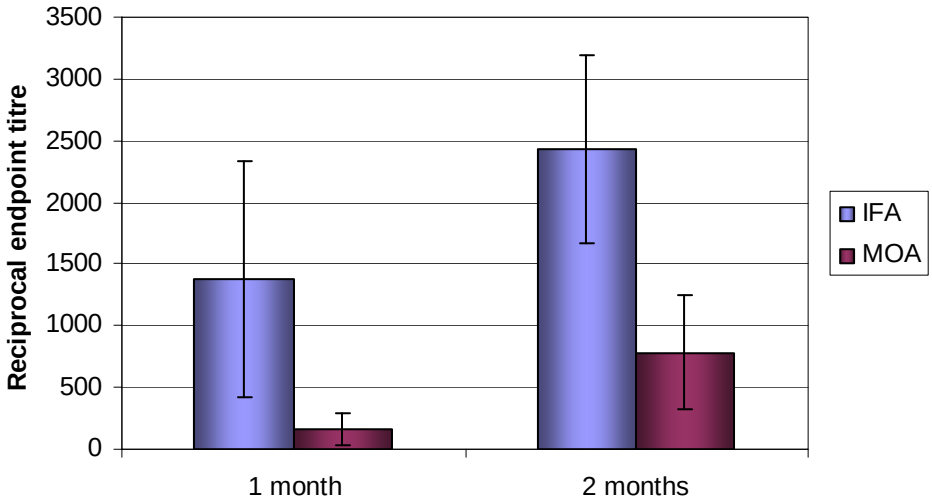


Figure 6.7. Graphical representation of the IgG anti-Tat reciprocal endpoint titres of 5 cattle vaccinated with CA/Tat-GST with the IFA and MOA adjuvants showing that the IFA adjuvant was associated with a greater humoral immune response to Tat.

Discussion

The vaccine trial was conducted to assess possible adverse effects under field conditions of the JDV CA/Tat-GST polyprotein vaccine and the duration of the vaccine specific antibody responses over a 12 month period. It had been anticipated that there might be an outbreak of Jembrana disease during the trial which might add to efficacy data, but this did not eventuate. The vaccinated and control cohorts included a large proportion of cattle that were pregnant. It is generally recommended to avoid vaccinating pregnant cattle during safety trials as the altered immune and hormonal status may interfere with assessing the vaccine safety and efficacy (OIE Manual of standard for diagnostic tests and vaccines, 2000).

All cattle were tested prior to the commencement of the trial to determine their infection status before vaccination. These assays detected antibody in 11.5% of the 200 cattle which is similar to what had been found previously in an epidemiological study in 1994 (Putra and Sulistyana, 1995; Wiryosuhanto, 1995). However, cattle less than 4 years of age were JDV antibody-negative indicating that JDV was not circulating in the young cattle and had not been for 4 years. It has been previously suggested that waning herd immunity may be an important factor in the development of Jembrana disease outbreaks which generally occur every 5-10 years (Ramachandran, 1995). It may be that once the prevalence of "immune" cattle (those that have been exposed to JDV previously) drop below a certain level then an outbreak can occur amongst the naïve cattle population (Wilcox, 1997). BIV has been shown to be transmitted *in utero* (Meas et al., 2002; Moody et al., 2002) and as JDV is presumed to persist in recovered cattle, the absence of seropositive young cattle in this study suggests that vertical transmission of JDV does not occur.

In the control group, some cattle fluctuated between being seropositive and seronegative during the 12 months of the trial (data not shown) but there was no significant occurrence of animals developing antibody during the 12 month period of the trial. The results in the control animals, with no significant occurrence of animals developing antibody during the 12 month period of the trial, indicates that Jembrana disease was not circulating in the cattle during the period of the trial. The intermittently seropositive status of some older cattle which was detected, has been reported previously in some field animals in JDV-endemic areas (Putra and

Sulistyana, 1995) and highlights the difficulties in diagnosing JDV-infected animals in the field.

Of the 100 cattle vaccinated with the fused JDV CA/Tat-GST protein in MOA, there were 5% that developed severe adverse reactions including anaphylactic shock and abortion although these adverse reactions also occurred in 3% of the control group. The rate of abortion in the vaccinated group (3/38, 7.9%) was not significantly higher than the rate in the control group (5.71%) which suggests that the abortions were not specific to the JDV CA/Tat-GST polyprotein vaccine. Abortion may have been associated with the movement of the pregnant cattle to a centralized vaccination site during hot and humid conditions at the time of vaccination. Two of the 3 abortions that occurred in the vaccinated group occurred in cattle with the same owner which suggests that the conditions under which the vaccination occurred rather than the actual vaccine may have been associated with the abortions. Regardless of the cause of the abortions, administration of the vaccine to pregnant cattle should be avoided as suggested by the OIE guidelines for vaccine and diagnostic reagents (Edwards, 2007).

There were also local reactions seen in the vaccinated and control cattle at the site of injection which were not seen in earlier laboratory trials with 120 cattle and where IFA was used. The anaphylactic shock and the local reactions were possibly due to the change of adjuvant from IFA to the MOA. While IFA is the preferred adjuvant of choice for laboratory scale development of vaccines as antigen persists in the injection site and is also transported efficiently to the lymphatics for presentation to dendritic cells and it is not associated with local or hypersensitivity reactions (Gupta et al., 1993), it is not economically viable for use in veterinary vaccinations (Gupta et al., 1993). Mineral oil adjuvants have been shown to induce good humoral and cell-mediated immune responses but have also been linked to local and hypersensitivity reactions similar to what was observed in the safety trial when used in conjunction with bacterial proteins (Aucouturier et al., 2001; Bowersock and Martin, 1999; Gupta et al., 1993). These local reactions may be avoided by the selection of alternative adjuvants or by the administration of less adjuvant per dose. Further studies are required to identify a low cost, safe and effective replacement for IFA.

All but 2 of the 100 vaccinated cattle had become CA and Tat antibody-positive 1 month after the initial vaccine dose and all had seroconverted to CA and Tat by 3 months post-vaccination and remained seropositive 12 months post-vaccination, in

nearly all cases with high residual antibody titres. There was a good response to both CA and Tat but the anti-Tat IgG responses were less than the anti-CA IgG responses. The 2 cattle that failed to elicit a CA or Tat antibody response 1 month after the first vaccine dose possibly did not receive the correct dose of vaccine in the initial vaccination as they responded well to the second vaccine dose, they developed high antibody titres 3 months post-vaccination but the antibody levels in these cattle declined faster than the remainder of the cattle and were nearly negative 12 months post-vaccination. The CA antibody titres obtained during this field trial were extremely high, much higher than has been previously reported with recombinant protein vaccines in humans, horses, monkeys and cattle where lower titres of between 1,000-40,000 were detected after 2 vaccinations, (Dupont et al., 2006; Estuningsih et al., 1997; Hammond et al., 1999b; Naik et al., 1997; Shokri and Jafarzadeh, 2001). The high titres obtained in the current study may have been associated with the large vaccine dose of 4 mg of protein/dose used compared to lower doses ranging from 10 μg for monkeys to 200 μg for horses (Hammond et al., 2000; Issel et al., 1992; Purcell et al., 2003). Further investigation of the effect of a reduced dose of the CA/Tat-GST polyprotein is required; this may not only be equally or even more efficacious, it would reduce costs of production.

Chapter 7. General discussion

Since the initial outbreak in 1964 of Jembrana disease in Bali, the disease has become endemic in the islands of Java and Sumatra and Indonesian Borneo (Kalimantan), with regular outbreaks every 4-6 years in the endemic regions (Hartaningsih et al., 1993). Attempts to control the spread of the disease by restriction of the movement of cattle from endemic area and ring vaccination around outbreaks with a tissue-derived inactivated virus vaccine has slowed but not prevented the spread of the disease. Jembrana disease causes significant financial loss to both the individual farmers and the government due to the direct effects of the disease and restrictions on trade of cattle between endemic and disease-free areas.

It is likely that successful control of JDV in Indonesia will only be achieved by development of effective immunosurveillance and a low cost alternative vaccine that would provide a long lived response and could be given to the majority of cattle in endemic and naïve areas in which the disease is detected. The current vaccine that is used is too expensive for widespread use and the development of an alternative vaccine has become a priority in Indonesia and led to the current project.

The first major objective of the studies reported in this thesis was the development of an improved recombinant protein antigen for serological studies and surveillance of JDV infections. This was needed because of the difficulty of producing, with the current equipment available in Indonesia, the whole virus antigen that is purified from the plasma of infected cattle. This objective was successfully achieved: a CA protein was developed that could be used in an ELISA and replace the whole virus antigen immunoassay. This recombinant CA antigen was relatively simple to produce and will allow laboratories in Indonesia to continually monitor JDV infection in endemic regions and in new outbreaks on a scale that was previously not possible with the whole virus immunoassay. The CA ELISA assay also had the advantage of being easy to produce with very high yields of pure recombinant protein. It was also considerably cheaper to produce than the whole virus antigen. Other recombinant protein antigens were developed and tested but were of lesser value. For example, it was found that all assays which included any part of the TM protein significantly reduced the sensitivity of the JDV assays, which is contrary to the findings published on other lentiviral diagnostic assays (Abed and Archambault, 2000; Abed et al., 1999; Calzolari et al.,

1995; de Andres et al., 2005; Rosati et al., 1994). The reason for this was not determined but it might be because of the unusual pathogenesis of Jembrana disease with an early acute disease process followed by apparent permanent immunity and no recurrence of disease. In other lentivirus infections, the humoral response to the CA is detected first after the acute phase and the TM response is generally detected after the later clinical onset of the ensuing chronic disease (Calzolari et al., 1995).

The second major objective of the studies reported was the development of an improved vaccine. Several approaches to achieving this were considered. An inactivated cell culture-derived whole virus vaccine, similar to that used for FIV, would have been an ideal replacement for the tissue-derived inactivated whole virus vaccine that has been used in Indonesia, but the virus has not been cultured *in vitro*. A recombinant protein vaccine was considered the next best alternative and the choice of expression system was driven by the expected production costs. The cost of a vaccine in Indonesia was an over-riding issue and as mammalian or insect cell expressed recombinant proteins have a poor yield and are expensive to produce, a bacterial expression system was chosen. The choice of which viral proteins to use was, however, limited by the use of the bacterial expression system chosen. Recombinant proteins expressed in bacteria do not undergo post-translational modifications and therefore JDV proteins that normally undergo significant posttranslational modifications such as the heavily glycosylated SU and TM would be different to the native proteins, which may explain why earlier attempts to use SU and TM proteins expressed in bacterial expression systems provided poor results (Ditcham, 2007).

Much of the work described in this thesis describes the scaling up of methods for the production of previous reagents and development of quality control procedures for the manufacture of a vaccine. Therefore there is an emphasis on the development of different methods of production for the vaccine rather than the testing of the vaccine.

In earlier Jembrana disease vaccine studies (Ditcham, 2007) there were poor yields and low purity of the individual CA-GST and the Tat-GST proteins that were used, possibly associated with toxicity issues in the bacterial expression system. These problems were overcome by fusing the individual *capsid* and *tat*1-expressed proteins into a single construct expressing a CA/Tat polyprotein. This seemed to remove the bacterial toxicity issues and allowed extensive optimisation of the methods used for

growth, induction and purification of the protein, resulting in very high levels of recombinant protein from bacterial cells and a high level of purity. This high level of recombinant protein recovery reduced the actual cost of production of the proteins per dose from A$2.18 per for 2 vaccine doses when individual CA-GST and Tat-GST proteins were produced to A$0.08 per 2 vaccine doses with the fused CA/Tat-GST polyprotein vaccine (2008 prices). This was a significant reduction when compared to the cost of the tissue-derived whole virus vaccine which cost A$2.66 for 2 vaccine doses. This should enable the implementation of a large scale vaccination program within Indonesia. The recombinant CA/Tat-GST polyprotein did not cause a significant increase in adverse reactions in cattle despite the very high dose of recombinant protein, and its use appeared to be relatively safe. Although there were some adverse reactions to vaccination with the CA/Tat-GST these could be reduced by following the recommendations (Chapter 6) that the vaccine be administered at 2 sites during vaccination and that pregnant cattle should not be vaccinated. In future, the protein per dose might be reduced without loss of efficacy and a change in the adjuvant could be considered to increase efficacy.

The use of the CA/Tat-GST polyprotein vaccine reported in Chapter 5 and 6 was not designed to be a stand alone analysis of the safety and efficacy of the vaccine but rather baseline studies and an indication on the direction that further development should take for a commercial vaccine company in Indonesia. The development of any vaccine needs to consider a variety of indices such as vaccine dose, the route of administration of the vaccine, the time between vaccinations, the time between final vaccination and effective immunity detected by challenge, identification of correlates of protection, whether to use homologous or heterologous challenge virus, the route of challenge and the challenge dose; all of these may affect the outcome of any trial (Dunham, 2006). There were several areas of concern from the trial conducted and reported in Chapter 5 that were unavoidable because of time and financial constraints but should be mentioned. The current studies must be considered as only part of an on-going process of vaccine development.

In the current experiments, a large dose of recombinant virus protein of 4 mg was incorporated into each vaccine dose. This large dose was selected after a series of previous vaccine trials where 3 vaccinations with 2 mg each of a relatively impure recombinant JDV CA-GST and Tat-GST were shown to reduce viral load and

ameliorate the disease after challenge (Ditcham, 2007). However, in the current investigations, vaccination 2 or 3 times with 4 mg of fused CA/Tat-GST polyprotein of high purity provided an extremely large dose compared to that used in other lentiviral particulate vaccines. Horses were vaccinated with 50-200 µg/dose of recombinant EIAV Env protein; similar urea/DTT recombinant protein vaccines for sheep contained 50 µg per dose, and other recombinant protein vaccines for cattle have contained 50-200 µg/dose (Hammond et al., 1999a; Issel et al., 1992; Purcell et al., 2003; Rothel et al., 1997; Shokri and Jafarzadeh, 2001; Wu et al., 2004). The large dose was used in the current experiment because of the earlier success obtained with high doses but it is realised that these vaccines reported by Ditcham (2007) were of low purity and effectively contained much less than 2 mg of each protein. The large dose of 4 mg was likely to be detrimental to vaccine efficacy (Fife et al., 2004) and the next stage of vaccine development should include a dose/response trial to determine the optimum dose whilst maintaining or even increasing vaccine efficacy.

Another factor of the current experiments that must be considered in the development of a Jembrana disease vaccine was the short periods of only 28 days between vaccinations and 28 day interval from the final vaccine dose until viral challenge. This short interval between vaccine doses was probably not ideal for an inclusion body-based vaccine but was selected to reduce the total holding time and cost of holding vaccinated cattle indoors. Short intervals between vaccinations have been effective for raising high titre antibody responses to vaccines but do not take into account the long-lived nature of insoluble recombinant protein vaccines or achieving an optimal cell-mediated response to such vaccines (Fanales-Belasio et al., 2002). Inclusion body vaccines generally take longer to be broken down *in vivo* (Hansson et al., 2000; Heath and Holcman, 1997; Rothel et al., 1997) and this short vaccination-challenge model failed to take into account cell-mediated immune responses which can take up to 8 months post-vaccination to develop (Fanales-Belasio et al., 2002; Hosie and Flynn, 1996). Further studies should take into account the time required for maturation of the humoral and cell-mediated immune responses to the viral antigens and compare efficacy of the different vaccine regimes especially in light of new research that the HIV-1 Tat protein broadens T-cell responses against the Gag and Env viral proteins (Gavioli et al., 2008; Gavioli et al., 2004). This would mean that responses to novel T

cell epitopes on the CA antigen important for protection against Jembrana disease may not have had enough time to mature between vaccination and challenge.

The challenge dose of about 1,000 ID_{50} of a heterologous JDV_{Pul01} strain was probably too high. It did, however, reliably induce Jembrana disease in all cattle (Ditcham, 2007; Stewart et al., 2005) and avoided the problem seen in previous trials with the individual protein vaccines, where infectious doses too small to infect all cattle were sometimes used and resulted in failed experiments. However, a challenge dose of 1,000 ID_{50} is extremely high and most groups have attempted to use lower challenge doses to mimic natural lentivirus infections (Finerty et al., 2000; Horton et al., 2002; Hosie and Flynn, 1996). A large challenge dose may considerably underestimate the effectiveness of the vaccine in field conditions. It is noted that when the tissue-derived inactivated virus vaccine was tested it was with a considerably lower challenge dose (400 ID_{50}) of a less pathogenic homologous JDV_{Tab87} strain (Hartaningsih et al., 2001). A recommendation of this study is to utilize a similar challenge conditions for testing the efficacy of the recombinant protein vaccine.

How to achieve this low challenge dose is a problem but one obvious solution is to utilise a standard quantified stored challenge dose of JDV. This could be achieved by storing virus in liquid nitrogen but a reliable source of liquid nitrogen in the laboratory in Bali has not been possible. This has led to infected spleen being stored at lower temperatures and then used to infect donor animals, with plasma from the donor animals on the second day of the febrile period used to infect cattle (Soeharsono et al., 1990). Problems of rapid quantification of the virus in the plasma from each donor animal made a consistent challenge dose a difficult problem. Additionally, the possible effect of multiple passage of the virus strains through donor animals could lead to variations in pathogenicity of the challenge strains.

Despite these issues, the recombinant CA/Tat-GST polyprotein vaccine that was developed showed promise in reducing the clinical signs of infection and partially protected the cattle from a heterologous challenge with JDV. The study described in Chapter 5 showed that the fused CA/Tat-GST polyprotein vaccine raised a greater humoral immune response and provided a similar level of clinical protection as the individual CA-GST plus Tat-GST which were shown to be efficacious at inducing a protective immunity (Ditcham, 2007).

References

2000. OIE Manual of Standards for Diagnostic Tests and Vaccines. Principles of validation of diagnostic assays for infectious disease, 15-23.

Abed, Y. , Archambault, D., 2000. A Viral Transmembrane Recombinant Protein-Based Enzyme-Linked Immunosorbent Assay for the Detection of Bovine Immunodeficiency Virus Infection. J Virol Methods 85, 109-16.

Abed, Y., St-Laurent, G., Zhang, H., Jacobs, R.M. , Archambault, D., 1999. Development of a Western Blot Assay for Detection of Bovine Immunodeficiency-Like Virus using Capsid and Transmembrane Envelope Proteins Expressed from Recombinant Baculovirus. Clin Diagn Lab Immunol 6, 168-172.

Adiwinata, R.T., 1968. Some informative notes on a rinderpest-like disease on the island of Bali. Bull Off Int Epizoot 69, 7-14.

Albini, A., Ferrini, S., Benelli, R., Sforzini, S., Giunciuglio, D., Aluigi, M.G., Proudfoot, A.E., Alouani, S., Wells, T.N., Mariani, G., Rabin, R.L., Farber, J.M. , Noonan, D.M., 1998. HIV-1 Tat protein mimicry of chemokines. Proc Natl Acad Sci U S A 95, 13153-8.

Andersson, S., da Silva, Z., Norrgren, H., Dias, F. , Biberfeld, G., 1997. Field evaluation of alternative testing strategies for diagnosis and differentiation of HIV-1 and HIV-2 infections in an HIV-1 and HIV-2-prevalent area. Aids 11, 1815-22.

Aoki, Y. , Tosato, G., 2004. HIV-1 Tat enhances Kaposi sarcoma-associated herpesvirus (KSHV) infectivity. Blood 104, 810-4.

Aoki, Y. , Tosato, G., 2007. Interactions between HIV-1 Tat and KSHV. Curr Top Microbiol Immunol 312, 309-26.

Aucouturier, J., Dupuis, L. , Ganne, V., 2001. Adjuvants designed for veterinary and human vaccines. Vaccine 19, 2666-72.

Badou, A., Bennasser, Y., Moreau, M., Leclerc, C., Benkirane, M. , Bahraoui, E., 2000. Tat protein of human immunodeficiency virus type 1 induces interleukin-10 in human peripheral blood monocytes: implication of protein kinase C-dependent pathway. J Virol 74, 10551-62.

Ball, J.M., Rushlow, K.E., Issel, C.J. , Montelaro, R.C., 1992. Detailed mapping of the antigenicity of the surface unit glycoprotein of equine infectious anemia virus by using synthetic peptide strategies. J Virol 66, 732-42.

Barboni, P., Thompson, I., Brownlie, J., Hartaningsih, N. , Collins, M.E., 2001. Evidence for the Presence of Two Bovine Lentiviruses in the Cattle Population of Bali. Vet Microbiol 80, 313-27.

Barboric, M., Taube, R., Nekrep, N., Fujinaga, K. , Peterlin, B.M., 2000. Binding of Tat to TAR and recruitment of positive transcription elongation factor b occur independently in bovine immunodeficiency virus. J Virol 74, 6039-44.

Baron, T., Betemps, D., Mallet, F., Cheynet, V., Levy, D. , Belli, P., 1997. Detection of Bovine Immunodeficiency-Like Virus Infection in Experimentally Infected Calves. Arch Virol 143, 181-189.

Bartz, S.R. , Emerman, M., 1999. Human immunodeficiency virus type 1 Tat induces apoptosis and increases sensitivity to apoptotic signals by up-regulating FLICE/caspase-8. J Virol 73, 1956-63.

Battles, J.K., Hu, M.Y., Rasmussen, L., Tobin, G.J. , Gonda, M.A., 1992. Immunological Characterization of the *gag* Gene Products of Bovine Immunodeficiency Virus. J Virol 66, 6868-6877.

Belliard, G., Hurtrel, B., Moreau, E., Lafont, B.A., Monceaux, V., Roques, B., Desgranges, C., Aubertin, A.M., Le Grand, R. , Muller, S., 2005. Tat-neutralizing versus Tat-protecting antibodies in rhesus macaques vaccinated with Tat peptides. Vaccine 23, 1399-407.

Benjouad, A., Mabrouk, K., Moulard, M., Gluckman, J.C., Rochat, H., Van Rietschoten, J. , Sabatier, J.M., 1993. Cytotoxic effect on lymphocytes of Tat from human immunodeficiency virus (HIV-1). FEBS Lett 319, 119-24.

Bennasser, Y., Badou, A., Tkaczuk, J. , Bahraoui, E., 2002. Signaling pathways triggered by HIV-1 Tat in human monocytes to induce TNF-alpha. Virology 303, 174-80.

Bennett, I.L., Jr. , Cluff, L.E., 1957. Bacterial pyrogens. Pharmacol Rev 9, 427-79.

Bertoni, G., Hertigb, C., Zahno, M., Vogt, H., Dufour, S., Cordano, P., Peterhans, E., Cheevers, W.P., Sonigo, P. , Pancinoc, G., 2000. B-cell epitopes of the envelope glycoprotein of caprine arthritis–encephalitis virus and antibody response in infected goats. J Gen Virol 81, 2929-2940.

Bertoni, G., Zahno, M.L., Zanoni, R., Vogt, H.R., Peterhans, E., Ruff, G., Cheevers, W.P., Sonigo, P. , Pancino, G., 1994. Antibody reactivity to the immunodominant epitopes of the caprine arthritis-encephalitis virus gp38 transmembrane protein associates with the development of arthritis. J Virol 68, 7139-47.

Bhatia, S., Sood, R., Bhatia, A.K., Pattnaik, B. , Pradhan, H.K., 2008. Development of a capsid based competitive inhibition enzyme-linked immunosorbent assay for detection of bovine immunodeficiency virus antibodies in cattle and buffalo serum. J Virol Methods 148, 218-25.

Blatteis, C.M., 2007. The onset of fever: new insights into its mechanism. Prog Brain Res 162, 3-14.

Blatteis, C.M. , Sehic, E., 1998. Cytokines and fever. Ann N Y Acad Sci 840, 608-18.

Boes, M., 2000. Role of natural and immune IgM antibodies in immune responses. Mol Immunol 37, 1141-9.

Bomsel, M., Heyman, M., Hocini, H., Lagaye, S., Belec, L., Dupont, C. , Desgranges, C., 1998. Intracellular neutralization of HIV transcytosis across tight epithelial barriers by anti-HIV envelope protein dIgA or IgM. Immunity 9, 277-87.

Borsutzky, S., Ebensen, T., Link, C., Becker, P.D., Fiorelli, V., Cafaro, A., Ensoli, B. , Guzman, C.A., 2006. Efficient systemic and mucosal responses against the HIV-1 Tat protein by prime/boost vaccination using the lipopeptide MALP-2 as adjuvant. Vaccine 24, 2049-56.

Bowersock, T.L. , Martin, S., 1999. Vaccine delivery to animals. Adv Drug Deliv Rev 38, 167-194.

Brady, J. , Kashanchi, F., 2005. Tat gets the "green" light on transcription initiation. Retrovirology 2, 69.

Brinkhof, J. , van Maanen, C., 2007. Evaluation of five enzyme-linked immunosorbent assays and an agar gel immunodiffusion test for detection of antibodies to small ruminant lentiviruses. Clin Vaccine Immunol 14, 1210-4.

Brown, E.W., Yuhki, N., Packer, C. , O'Brien, S.J., 1994. A lion lentivirus related to feline immunodeficiency virus: epidemiologic and phylogenetic aspects. J Virol 68, 5953-68.

Budiarso, I.T. , Hardjosworo, S., 1976. Jembrana disease in Bali cattle. Aust Vet J 52, 97.

Burgers, W.A., van Harmelen, J.H., Shephard, E., Adams, C., Mgwebi, T., Bourn, W., Hanke, T., Williamson, A.L. , Williamson, C., 2006. Design and preclinical

evaluation of a multigene human immunodeficiency virus type 1 subtype C DNA vaccine for clinical trial. J Gen Virol 87, 399-410.
Burkala, E.J., Narayani, I., Hartaningsih, N., Kertayadnya, G., Berryman, D.I. , Wilcox, G.E., 1998. Recombinant Jembrana disease virus proteins as antigens for the detection of antibody to bovine lentiviruses. J Virol Methods 74, 39-46.
Burkala, E.J., Narayani, I. , Wilcox, G.E., 1995. Expression of Recombinant Jembrana Disease Virus *env* Protein in *Escherichia.coli*. Jembrana Disease and the Bovine Lentivirus, 150-158.
Burkhard, M.J. , Dean, G.A., 2003. Transmission and immunopathogenesis of FIV in cats as a model for HIV. Curr HIV Res 1, 15-29.
Butler, J.E., 1983. Bovine immunoglobulins: an augmented review. Vet Immunol Immunopathol 4, 43-152.
Calzolari, M., Young, E., Cox, D., Davis, D. , Lutz, H., 1995. Serological diagnosis of feline immunodeficiency virus infection using recombinant transmembrane glycoprotein. Vet Immunol Immunopathol 46, 83-92.
Campbell, R.S.F., 1995. The Comparative Pathology of the Lentivirus. Jembrana Disease and the Bovine Lentivirus, 115-123.
Caputo, A., Gavioli, R. , Ensoli, B., 2004. Recent advances in the development of HIV-1 Tat-based vaccines. Curr HIV Res 2, 357-76.
Carpenter, S., Miller, L.D., Alexanderson, S., Whetstone, C.A., VanDerMaaten, M.J., Viuff, B., Wannemuehler, Y., Miller, J.A. , Roth, J.A., 1992. Characterization of Early Pathogenic Effects after Experimental Infection of Calves with Bovine Immunodeficiency-Like Virus. J Virol 66, 1074-1083.
Carpenter, S., Vaughn, E.M., Yang, J., Baccam, P., Roth, J.A. , Wannemuehler, Y., 2000. Antigenic and Genetic Stability of Bovine Immunodeficiency Virus during Long-Term Persistence in Cattle Experimentally Infected with the BIV(R29) Isolate. Journal of General Virology 81, 1463-1472.
Carrio, M.M., Corchero, J.L. , Villaverde, A., 1999. Proteolytic digestion of bacterial inclusion body proteins during dynamic transition between soluble and insoluble forms. Biochim Biophys Acta 1434, 170-6.
Carrio, M.M. , Villaverde, A., 2001. Protein aggregation as bacterial inclusion bodies is reversible. FEBS Lett 489, 29-33.
Carrio, M.M. , Villaverde, A., 2002. Construction and deconstruction of bacterial inclusion bodies. J Biotechnol 96, 3-12.
Casadevall, A. , Pirofski, L.A., 2003. Antibody-mediated regulation of cellular immunity and the inflammatory response. Trends Immunol 24, 474-8.
Casadevall, A. , Pirofski, L.A., 2004. New concepts in antibody-mediated immunity. Infect Immun 72, 6191-6.
Caselli, E., Betti, M., Grossi, M.P., Balboni, P.G., Rossi, C., Boarini, C., Cafaro, A., Barbanti-Brodano, G., Ensoli, B. , Caputo, A., 1999. DNA immunization with HIV-1 tat mutated in the trans activation domain induces humoral and cellular immune responses against wild-type Tat. J Immunol 162, 5631-8.
Cavacini, L.A., Kuhrt, D., Duval, M., Mayer, K. , Posner, M.R., 2003. Binding and neutralization activity of human IgG1 and IgG3 from serum of HIV-infected individuals. AIDS Res Hum Retroviruses 19, 785-92.
Celer, V., Jr. , Celer, V., 2001. Detection of antibodies to ovine lentivirus using recombinant capsid and transmembrane proteins. J Vet Med B Infect Dis Vet Public Health 48, 89-95.
Chadwick, B.B.V.M.S., B.Sc. (Hons). 1995. Genetic Studies of Jembrana Disease Virus, Philosophy at Murdoch University, Murdoch University, Perth, pp. 112.

Chadwick, B.D., Coelen, R.J., Sammels, L.M., Kertayadnya, G. , Wilcox, G.E., 1995a. Genomic Sequence Analysis Identifies Jembrana Disease Virus as a New Bovine Lentivirus. J Gen Virol 76, 189-192.
Chadwick, B.D., Coelen, R.J., Wilcox, G.E., Sammels, L.M. , Kertayadnya, G., 1995b. Nucleotide Seqeunce Analysis of Jembrana Disease Virus: a Bovine Lentivirus Associated with an Acute Disease Syndrome. J Gen Virol 76, 1637-1650.
Chadwick, B.J., Desport, M., Brownlie, J., Wilcox, G.E. , Dharma, D.M., 1998. Detection of Jembrana Disease Virus in Spleen, Lymph Nodes, Bone Marrow and other Tissues by in Situ Hybridization of Paraffin-Embedded Sections. J Gen Virol 79 (Pt 1), 101-106.
Chakrabarti, L., Isola, P., Cumont, M.C., Claessens-Maire, M.A., Hurtrel, M., Montagnier, L. , Hurtrel, B., 1994. Early Stages of Simian Immunodeficiency Virus Infection in Lymph Nodes. Evidence for High Viral Load and Successive Populations of Target Cells. Am J Pathol 144, 1226-37.
Chang, H.C., Samaniego, F., Nair, B.C., Buonaguro, L. , Ensoli, B., 1997. HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region. Aids 11, 1421-31.
Cheevers, W.P., Knowles, D.P., McGuire, T.C., Cunningham, D.R., Adams, D.S. , Gorham, J.R., 1988. Chronic Disease in Goats Orally Infected with Two Isolates of the Caprine Arthritis-Encephalitis Lentivirus. Lab Invest 58, 510-7.
Chen, H., Wilcox, G., Kertayadnya, G., Wood, C., 1999. Characterization of the Jembrana disease virus tat gene and the cis- and trans-regulatory elements in its long terminal repeats. J Virol 73, 658-66.
Chen, Y. , Varani, G., 2005. Protein families and RNA recognition. Febs J 272, 2088-97.
Chien, P.C., Jr., Cohen, S., Tuen, M., Arthos, J., Chen, P.D., Patel, S. , Hioe, C.E., 2004. Human immunodeficiency virus type 1 evades T-helper responses by exploiting antibodies that suppress antigen processing. J Virol 78, 7645-52.
Chitsulo, L., Engels, D., Montresor, A. , Savioli, L., 2000. The global status of schistosomiasis and its control. Acta Trop 77, 41-51.
Chong, Y.H., Ball, J.M., Issel, C.J., Montelaro, R.C. , Rushlow, K.E., 1991. Analysis of equine humoral immune responses to the transmembrane envelope glycoprotein (gp45) of equine infectious anemia virus. J Virol 65, 1013-8.
Chung, C., Mealey, R.H. , McGuire, T.C., 2004. CTL from EIAV carrier horses with diverse MHC class I alleles recognize epitope clusters in Gag matrix and capsid proteins. Virology 327, 144-54.
Chung, C., Mealey, R.H. , McGuire, T.C., 2005. Evaluation of high functional avidity CTL to Gag epitope clusters in EIAV carrier horses. Virology 342, 228-39.
Cole, K.S., Murphey-Corb, M., Narayan, O., Joag, S.V., Shaw, G.M. , Montelaro, R.C., 1998. Common Themes of Antibody Maturation to Simian Immunodeficiency Virus, Simian-Human Immunodeficiency Virus and Human Immunodeficiency Virus Type1. J Virol 72, 7852-7859.
Coleman, J.K., Pu, R., Martin, M., Sato, E. , Yamamoto, J.K., 2005. HIV-1 p24 vaccine protects cats against feline immunodeficiency virus infection. Aids 19, 1457-66.
Conaldi, P.G., Bottelli, A., Baj, A., Serra, C., Fiore, L., Federico, G., Bussolati, B. , Camussi, G., 2002. Human immunodeficiency virus-1 tat induces

hyperproliferation and dysregulation of renal glomerular epithelial cells. Am J Pathol 161, 53-61.
Cotran, R.S., Kumar, V. , Collins, T., 1999. Robbins Pathologic Basis of Disease. W.B Saunders, Philadelphia.
Dandekar, D.H., Ganesh, K.N. , Mitra, D., 2004. HIV-1 Tat directly binds to NFkappaB enhancer sequence: role in viral and cellular gene expression. Nucleic Acids Res 32, 1270-8.
Dawson, M., 1987. Pathogenesis of Maedi-Visna. Vet Rec 120, 451-4.
de Andres, D., Klein, D., Watt, N.J., Berriatua, E., Torsteinsdottir, S., Blacklaws, B.A. , Harkiss, G.D., 2005. Diagnostic tests for small ruminant lentiviruses. Vet Microbiol 107, 49-62.
DeMartini, J.C., Halsey, W., Boshoff, C., York, D. , Howell, M.D., 1999. Comparison of a maedi-visna virus CA-TM fusion protein ELISA with other assays for detecting sheep infected with North American ovine lentivirus strains. Vet Immunol Immunopathol 71, 29-40.
Deng, G., Qiao, W., Su, Y., Sha, R., Geng, Y. , Chen, Q., 2006. Internalization of Jembrana disease virus Tat: Possible pathway and implication. Virus Res.
Desport, M., Stewart, M.E., Mikosza, A.S., Sheridan, C.A., Peterson, S.E., Chavand, O., Hartaningsih, N. , Wilcox, G.E., 2007. Sequence analysis of Jembrana disease virus strains reveals a genetically stable lentivirus. Virus Res 126, 233-44.
Desport, M., Stewart, M.E., Sheridan, C.A., Ditcham, W.G., Setiyaningsih, S., Tenaya, W.M., Hartaningsih, N. , Wilcox, G.E., 2005. Recombinant Jembrana disease virus gag proteins identify several different antigenic domains but do not facilitate serological differentiation of JDV and nonpathogenic bovine lentiviruses. J Virol Methods 124, 135-42.
Dharma, D.M., 1995. The Pathology of Jembrana Disease. Jembrana Disease and the Bovine Lentivirus, 26-28.
Ditcham, W. 2007. The Development of Recombinant Vaccines Against Jembrana Disease, Veterinary and Biomedical Sciences, Vol. PhD, Murdoch University, Perth, pp. 198.
Dufour, S., Zahno, M.L., Vogt, H.R., Peterhans, E. , Bertoni, G., 2002. Production of monospecific antibody to immunodominant epitopes of the caprine arthritis encephalitis virus transmembrane glycoprotein and analysis of their activity in vitro. Intervirology 45, 177-82.
Dunham, S.P., 2006. Lessons from the cat: development of vaccines against lentiviruses. Vet Immunol Immunopathol 112, 67-77.
Dunham, S.P., Bruce, J., MacKay, S., Golder, M., Jarrett, O. , Neil, J.C., 2006. Limited efficacy of an inactivated feline immunodeficiency virus vaccine. Vet Rec 158, 561-2.
Dupont, J., Altclas, J., Lepetic, A., Lombardo, M., Vazquez, V., Salgueira, C., Seigelchifer, M., Arndtz, N., Antunez, E., von Eschen, K. , Janowicz, Z., 2006. A controlled clinical trial comparing the safety and immunogenicity of a new adjuvanted hepatitis B vaccine with a standard hepatitis B vaccine. Vaccine 24, 7167-74.
Edwards, S., 2007. OIE standards for vaccines and future trends. Rev Sci Tech 26, 373-8.
Egan, M.A., 2004. Current prospects for the development of a therapeutic vaccine for the treatment of HIV type 1 infection. AIDS Res Hum Retroviruses 20, 794-806.

Engels, D., Chitsulo, L., Montresor, A. , Savioli, L., 2002. The global epidemiological situation of schistosomiasis and new approaches to control and research. Acta Trop 82, 139-46.
Enoe, C., Georgiadis, M.P. , Johnson, W.O., 2000. Estimation of sensitivity and specificity of diagnostic tests and disease prevalence when the true disease state is unknown. Prev Vet Med 45, 61-81.
Estuningsih, S.E., Smooker, P.M., Wiedosari, E., Widjajanti, S., Vaiano, S., Partoutomo, S. , Spithill, T.W., 1997. Evaluation of antigens of Fasciola gigantica as vaccines against tropical fasciolosis in cattle. Int J Parasitol 27, 1419-28.
Fanales-Belasio, E., Cafaro, A., Cara, A., Negri, D.R., Fiorelli, V., Butto, S., Moretti, S., Maggiorella, M.T., Baroncelli, S., Michelini, Z., Tripiciano, A., Sernicola, L., Scoglio, A., Borsetti, A., Ridolfi, B., Bona, R., Ten Haaft, P., Macchia, I., Leone, P., Pavone-Cossut, M.R., Nappi, F., Vardas, E., Magnani, M., Laguardia, E., Caputo, A., Titti, F. , Ensoli, B., 2002. HIV-1 Tat-based vaccines: from basic science to clinical trials. DNA Cell Biol 21, 599-610.
Fauci, A.S., Schnittman, S.M., Poli, G., Koenig, S. , Pantaleo, G., 1991. NIH conference. Immunopathogenic mechanisms in human immunodeficiency virus (HIV) infection. Ann Intern Med 114, 678-93.
Fauquet, C.M. , Mayo, M.A., 2001. The 7th ICTV report. Arch Virol 146, 189-94.
Feliu, J.X., Cubarsi, R. , Villaverde, A., 1998. Optimized release of recombinant proteins by ultrasonication of E. coli cells. Biotechnol Bioeng 58, 536-40.
Fife, K.H., Wheeler, C.M., Koutsky, L.A., Barr, E., Brown, D.R., Schiff, M.A., Kiviat, N.B., Jansen, K.U., Barber, H., Smith, J.F., Tadesse, A., Giacoletti, K., Smith, P.R., Suhr, G. , Johnson, D.A., 2004. Dose-ranging studies of the safety and immunogenicity of human papillomavirus Type 11 and Type 16 virus-like particle candidate vaccines in young healthy women. Vaccine 22, 2943-52.
Finerty, S., Stokes, C.R., Gruffydd-Jones, T.J., Hillman, T.J., Reeves, N.A., Whiting, C.V., Schaaper, W.M., Dalsgaard, K. , Harbour, D.A., 2000. Mucosal immunization with experimental feline immunodeficiency virus (FIV) vaccines induces both antibody and T cell responses but does not protect against rectal FIV challenge. Vaccine 18, 3254-65.
Flaming, K., van der Maaten, M., Whetstone, C., Carpenter, S., Frank, D. , Roth, J., 1993. Effect of Bovine Immunodeficiency-Like Virus Infection on Immune Function in Experimentally Infected Cattle. Vet Immunol Immunopathol 36, 91-105.
Fleiss, J.L., 1981. Statistical methods for rates and proportions. Wiley, New York.
Flint, S.J., Enquist, L.W., Krug, R.M., Racaniello, V.R. , Skalka, A.M., 2000. Principles of Virology. ASM press, Washington.
Foil, L.D., Meek, C.L., Adams, W.V. , Issel, C.J., 1983. Mechanical Transmission of Equine Infectious Anemia Virus by Deer Flies (*Chrysops flavidus*) and Stable Flies (*Stomoxys calcitrans*). Am J Vet Res 44, 155-6.
Foley, G. 1998. An Overview of the Molecular Phylogeny of Lentiviruses, HIV Sequence Compendium 2000, Vol. Los Alamos, Theoretical Biology and Biophysics Group, pp. 35-43.
Friedhoff, P., Matzen, S.E., Meiss, G. , Pingoud, A., 1996. A quantitative microtiter plate nuclease assay based on ethidium/DNA fluorescence. Anal Biochem 240, 283-8.
Fulcher, A.J. , Jans, D.A., 2003. The HIV-1 Tat transactivator protein: a therapeutic target? IUBMB Life 55, 669-80.

Gabali, A.M., Anzinger, J.J., Spear, G.T. , Thomas, L.L., 2004. Activation by inflammatory stimuli increases neutrophil binding of human immunodeficiency virus type 1 and subsequent infection of lymphocytes. J Virol 78, 10833-6.
Gaines Das, R.E., Brugger, P., Patel, M., Mistry, Y. , Poole, S., 2004. Monocyte activation test for pro-inflammatory and pyrogenic contaminants of parenteral drugs: test design and data analysis. J Immunol Methods 288, 165-77.
Gallaher, W.R., Ball, J.M., Garry, R.F., Griffin, M.C. , Montelaro, R.C., 1989. A General Model for the Transmembrane Proteins of HIV and other Retroviruses. AIDS Res Hum Retroviruses 5, 431-40.
Gallaher, W.R., Ball, J.M., Garry, R.F., Martin-Amedee, A.M. , Montelaro, R.C., 1995. A General Model for the Surface Glycoproteins of HIV and other Retroviruses. AIDS Res Hum Retroviruses 11, 191-202.
Garber, M.E., Wei, P., KewalRamani, V.N., Mayall, T.P., Herrmann, C.H., Rice, A.P., Littman, D.R. , Jones, K.A., 1998. The interaction between HIV-1 Tat and human cyclin T1 requires zinc and a critical cysteine residue that is not conserved in the murine CycT1 protein. Genes Dev 12, 3512-27.
Garvey, K.J., Oberste, M.S., Elser, J.E., Braun, M.J. , Gondo, M.A., 1990. Nucleotide Sequence and Genome organization of Biologically Active Proviruses of the Bovine Immunodeficiency-like Virus. Virology 175, 391-409.
Gavioli, R., Cellini, S., Castaldello, A., Voltan, R., Gallerani, E., Gagliardoni, F., Fortini, C., Cofano, E.B., Triulzi, C., Cafaro, A., Srivastava, I., Barnett, S., Caputo, A. , Ensoli, B., 2008. The Tat protein broadens T cell responses directed to the HIV-1 antigens Gag and Env: Implications for the design of new vaccination strategies against AIDS. Vaccine 26, 727-37.
Gavioli, R., Gallerani, E., Fortini, C., Fabris, M., Bottoni, A., Canella, A., Bonaccorsi, A., Marastoni, M., Micheletti, F., Cafaro, A., Rimessi, P., Caputo, A. , Ensoli, B., 2004. HIV-1 tat protein modulates the generation of cytotoxic T cell epitopes by modifying proteasome composition and enzymatic activity. J Immunol 173, 3838-43.
Gekko, K. , Timasheff, S.N., 1981. Thermodynamic and kinetic examination of protein stabilization by glycerol. Biochemistry 20, 4677-86.
Georgsson, G., 1994. Neuropathologic Aspects of Lentiviral Infections. Ann N Y Acad Sci 724, 50-67.
Giacca, M., 2005. HIV-1 Tat, apoptosis and the mitochondria: a tubulin link? Retrovirology 2, 7.
Goldstein, G., 1996. HIV-1 Tat protein as a potential AIDS vaccine. Nat Med 2, 960-4.
Gonda, M.A., 1992. Bovine Immunodeficiency Virus. AIDS 6, 759-776.
Gonda, M.A., Braun, M.J., Carter, S.G., Kost, T.A., Bess, J.W., Jr., Arthur, L.O. , Van der Maaten, M.J., 1987. Characterization and Molecular Cloning of a Bovine Lentivirus Related to Human Immunodeficiency Virus. Nature 330, 388-91.
Gonda, M.A., Luther, D.G., Fong, S.E. , Tobin, G.J., 1994. Bovine Immunodeficiency Virus:Molecular Biology and Virus-Host Interactions. Virus Res 32, 155-181.
Gonzalez-Montalban, N., Carrio, M.M., Cuatrecasas, S., Aris, A. , Villaverde, A., 2005. Bacterial inclusion bodies are cytotoxic in vivo in absence of functional chaperones DnaK or GroEL. J Biotechnol 118, 406-12.
Gotch, F., Rutebemberwa, A., Jones, G., Imami, N., Gilmour, J., Kaleebu, P. , Whitworth, J., 2000. Vaccines for the control of HIV/AIDS. Trop Med Int Health 5, A16-21.

Greiner, M. , Gardner, I.A., 2000. Epidemiologic issues in the validation of veterinary diagnostic tests. Prev Vet Med 45, 3-22.
Greiner, M., Pfeiffer, D., Smith, R.D., 2000. Principles and practical application of the receiver-operating characteristic analysis for diagnostic tests. Prev Vet Med 45, 23-41.
Greiner, M., Sohr, D. , Gobel, P., 1995. A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. J Immunol Methods 185, 123-32.
Gupta, R.K., Relyveld, E.H., Lindblad, E.B., Bizzini, B., Ben-Efraim, S. , Gupta, C.K., 1993. Adjuvants--a balance between toxicity and adjuvanticity. Vaccine 11, 293-306.
Hammond, S.A., Cook, S.J., Falo, L.D., Jr., Issel, C.J. , Montelaro, R.C., 1999a. A particulate viral protein vaccine reduces viral load and delays progression to disease in immunized ponies challenged with equine infectious anemia virus. Virology 254, 37-49.
Hammond, S.A., Cook, S.J., Lichtenstein, D.L., Issel, C.J. , Montelaro, R.C., 1997. Maturation of the cellular and humoral immune responses to persistent infection in horses by equine infectious anemia virus is a complex and lengthy process. J Virol 71, 3840-52.
Hammond, S.A., Li, F., McKeon, B.M., Sr., Cook, S.J., Issel, C.J. , Montelaro, R.C., 2000. Immune responses and viral replication in long-term inapparent carrier ponies inoculated with equine infectious anemia virus. J Virol 74, 5968-81.
Hammond, S.A., Raabe, M.L., Issel, C.J. , Montelaro, R.C., 1999b. Evaluation of antibody parameters as potential correlates of protection or enhancement by experimental vaccines to equine infectious anemia virus. Virology 262, 416-30.
Hansson, M., Nygren, P.A. , Stahl, S., 2000. Design and production of recombinant subunit vaccines. Biotechnol Appl Biochem 32 (Pt 2), 95-107.
Hartaningsih, N. 1993. Aetiological and Immunological Aspects of Jembrana Disease in Bali Cattle, Vetinary Studies, Murdoch University, Perth, pp. 250.
Hartaningsih, N., Dharma, D.M., Soeharsono, S. , Wilcox, G.E., 2001. The Induction of a Protective Immunity Against Jembrana Disease in Cattle by Vaccination with Inactivated Tissue-Derived Virus Antigens. Vet Immunol Immunopathol 78, 163-76.
Hartaningsih, N., Soeharsono, S., Dharma, D.M.N., Kertayadnya, G., Sulistyana, K., Budiantono, A. , Wilcox, G.E., 1995a. The Development and Use of a Vaccine for the Control of Jembrana Disease. Jembrana Disease and the Bovine Lentivirus, 85-89.
Hartaningsih, N., Sulistyana, K. , Wilcox, G.E., 1995b. Serological test for JDV Antibodies and Antibody Response of Infected Cattle. Jembrana Disease and the Bovine Lentivirus, 79-82.
Hartaningsih, N., Wilcox, G.E., Dharma, D.M. , Soetrisno, M., 1993. Distribution of Jembrana disease in cattle in Indonesia. Vet Microbiol 38, 23-9.
Hartaningsih, N., Wilcox, G.E., Kertayadnya, G. , Astawa, M., 1994. Antibody response to Jembrana disease virus in Bali cattle. Vet Microbiol 39, 15-23.
Heath, D.D. , Holcman, B., 1997. Vaccination against echinococcus in perspective. Acta Trop 67, 37-41.
Heaton, P.R., Johnstone, P. , Brownlie, J., 1998. Investigation of the cellular tropism of bovine immunodeficiency-like virus. Res Vet Sci 65, 33-40.

Hecht, F.M., Busch, M.P., Rawal, B., Webb, M., Rosenberg, E., Swanson, M., Chesney, M., Anderson, J., Levy, J. , Kahn, J.O., 2002. Use of laboratory tests and clinical symptoms for identification of primary HIV infection. Aids 16, 1119-29.

Hodinka, R.L., Nagashunmugam, T. , Malamud, D., 1998. Detection of human immunodeficiency virus antibodies in oral fluids. Clin Diagn Lab Immunol 5, 419-26.

Hofmann-Lehmann, R., Holznagel, E., Aubert, A., Bauer-Pham, K. , Lutz, H., 1995. FIV vaccine studies. II. Clinical findings, hematological changes and kinetics of blood lymphocyte subsets. Vet Immunol Immunopathol 46, 115-25.

Horton, H., Vogel, T., O'Connor, D., Picker, L. , Watkins, D.I., 2002. Analysis of the immune response and viral evolution during the acute phase of SIV infection. Vaccine 20, 1927-32.

Hosie, M.J. , Flynn, J.N., 1996. Feline immunodeficiency virus vaccination: characterization of the immune correlates of protection. J Virol 70, 7561-8.

Hosie, M.J., Osborne, R., Reid, G., Neil, J.C. , Jarrett, O., 1992. Enhancement after feline immunodeficiency virus vaccination. Vet Immunol Immunopathol 35, 191-7.

Hosie, M.J., Osborne, R., Yamamoto, J.K., Neil, J.C. , Jarrett, O., 1995. Protection against homologous but not heterologous challenge induced by inactivated feline immunodeficiency virus vaccines. J Virol 69, 1253-5.

Hotzel, I. , Cheevers, W.P., 2000. Sequence similarity between the envelope surface unit (SU) glycoproteins of primate and small ruminant lentiviruses. Virus Res 69, 47-54.

Houwers, D.J. , Nauta, I.M., 1989. Immunoblot analysis of the antibody response to ovine lentivirus infections. Vet Microbiol 19, 127-39.

Hovden, A.O. 2001. The Influence of CXCR4 on Maedi Visna Virus Induced Syncytium., Molecular biology and Centre for Research in Virology, University of Bergen, pp. 77.

Huang, Y.T., Wright, A., Gao, X., Kulick, L., Yan, H. , Lamm, M.E., 2005. Intraepithelial cell neutralization of HIV-1 replication by IgA. J Immunol 174, 4828-35.

Isaacson, J.A., Roth, J.A., Wood, C. , Carpenter, S., 1995. Loss of Gag-specific antibody reactivity in cattle experimentally infected with bovine immunodeficiency-like virus. Viral Immunol 8, 27-36.

Issel, C.J., Horohov, D.W., Lea, D.F., Adams, W.V., Jr., Hagius, S.D., McManus, J.M., Allison, A.C. , Montelaro, R.C., 1992. Efficacy of inactivated whole-virus and subunit vaccines in preventing infection and disease caused by equine infectious anemia virus. J Virol 66, 3398-408.

Jacobs, R.M., 1995. Studies of Bovine Lentivirus Infection in Sheep. Jembrana Disease and the Bovine Lentivirus, 133-137.

Janssen, R.S., Satten, G.A., Stramer, S.L., Rawal, B.D., O'Brien, T.R., Weiblen, B.J., Hecht, F.M., Jack, N., Cleghorn, F.R., Kahn, J.O., Chesney, M.A. , Busch, M.P., 1998. New testing strategy to detect early HIV-1 infection for use in incidence estimates and for clinical and prevention purposes. JAMA 280, 42-8.

Jeang, K.T., Xiao, H. , Rich, E.A., 1999. Multifaceted activities of the HIV-1 transactivator of transcription, Tat. J Biol Chem 274, 28837-40.

Jolly, P.E., Huso, D.L., Sheffer, D. , Narayan, O., 1989. Modulation of lentivirus replication by antibodies: Fc portion of immunoglobulin molecule is essential

for enhancement of binding, internalization, and neutralization of visna virus in macrophages. J Virol 63, 1811-3.
Jones, K.A. , Peterlin, B.M., 1994. Control of RNA initiation and elongation at the HIV-1 promoter. Annu Rev Biochem 63, 717-43.
Kannagi, M., Kiyotaki, M., Desrosiers, R.C., Reimann, K.A., King, N.W., Waldron, L.M. , Letvin, N.L., 1986. Humoral immune responses to T cell tropic retrovirus simian T lymphotropic virus type III in monkeys with experimentally induced acquired immune deficiency-like syndrome. J Clin Invest 78, 1229-36.
Kashanchi, F., Agbottah, E.T., Pise-Masison, C.A., Mahieux, R., Duvall, J., Kumar, A. , Brady, J.N., 2000. Cell cycle-regulated transcription by the human immunodeficiency virus type 1 Tat transactivator. J Virol 74, 652-60.
Keirnan, R.E., Ono, A., Englund, G. , Freed, E.O., 1998. Role of Matrix in an Early Postentry Step in the Human Immunodeficiency Virus Type 1 Life Cycle. J Virol 72, 4116-4126.
Kerkau, T., Bacik, I., Bennink, J.R., Yewdell, J.W., Hunig, T., Schimpl, A. , Schubert, U., 1997. The human immunodeficiency virus type 1 (HIV-1) Vpu protein interferes with an early step in the biosynthesis of major histocompatibility complex (MHC) class I molecules. J Exp Med 185, 1295-305.
Kertayadnya, G., Soeharsono, S., Hartaningsih, N and Wilcox, G.E, 1995. Physiochemical Characteristics of a Virus Associated with Jembrana Disease. Jembrana Disease and the Bovine Lentivirus, 43-48.
Kertayadnya, G., Sulistyana, K. , Budianto, A., 1995. Anti-JDV Monoclonal Antibodies and Immunocytochemical Tests. Jembrana Disease and the Bovine Lentivirus, 61-65.
Kertayadnya, G., Wilcox, G.E., Soeharsono, S., Hartaningsih, N., Coelen, R.J., Cook, R.D., Collins, M.E. , Brownlie, J., 1993. Characteristics of a Retrovirus Associated with Jembrana Disease in Bali Cattle. J Gen Virol 74, 1765-1773.
Klebanoff, S.J. , Headley, C.M., 1999. Activation of the human immunodeficiency virus-1 long terminal repeat by respiratory burst oxidants of neutrophils. Blood 93, 350-6.
Kong, W.P., Huang, Y., Yang, Z.Y., Chakrabarti, B.K., Moodie, Z. , Nabel, G.J., 2003. Immunogenicity of multiple gene and clade human immunodeficiency virus type 1 DNA vaccines. J Virol 77, 12764-72.
Kopito, R.R., 2000. Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol 10, 524-30.
Kwang, J., Keen, J., Cutlip, R.C. , Littledike, E.T., 1993. Evaluation of an ELISA for detection of ovine progressive pneumonia antibodies using a recombinant transmembrane envelope protein. J Vet Diagn Invest 5, 189-93.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-5.
Langley, R.J., Hirsch, V.M., O'Brien, S.J., Adger-Johnson, D., Goeken, R.M. , Olmsted, R.A., 1994. Nucleotide sequence analysis of puma lentivirus (PLV-14): genomic organization and relationship to other lentiviruses. Virology 202, 853-64.
LaVallie, E.R. , McCoy, J.M., 1995. Gene fusion expression systems in Escherichia coli. Curr Opin Biotechnol 6, 501-6.
Letvin, N.L., 2002. Strategies for an HIV vaccine. J Clin Invest 110, 15-20.
Levin, J. , Bang, F.B., 1964. The Role Of Endotoxin In The Extracellular Coagulation Of Limulus Blood. Bull Johns Hopkins Hosp 115, 265-74.

Levy, J.A., ed, 1993a. The Viruses. The Retroviridae 1, Vol. 1. Plenum Press, New York.
Levy, J.A., ed, 1993b. The Viruses. The Retroviridae 2, Vol. 2. Plenum Press, New York.
Levy, J.A., ed, 1993c. The Viruses. The Retroviridae 3, Vol. 3. Plenum press, New York.
Levy, J.A., ed, 1993d. The Viruses. The Retroviridae 4, Vol. 4. Plenum Press, New York.
Lew, A.E., Bock, R.E., Miles, J., Cuttel, L.B., Steer, P. , Nadin-Davis, S.A., 2003. Sensitive and Specific Detection of Bovine Immunodeficiency Virus and Bovine Syncytial Virus by 5' *Taq* Nucleasae Assays with Flourescent 3' Minor Groove Binder-DNA Probes. J Virol Methods 116, 1-9.
Lewis, J. 2004. Characterization of the antibody response against recombinant proteins of the Jembrana disease virus (JDV), Veterinary and Biomedical Science, Murdoch University, Perth.
Lilie, H., Schwarz, E. , Rudolph, R., 1998. Advances in refolding of proteins produced in E. coli. Curr Opin Biotechnol 9, 497-501.
Liu, Z.Q., Sheridan, D. , Wood, C., 1992. Identification and Characterization of the Bovine Immunodeficiency-Like Virus *tat* Gene. J Virol 66, 5137-5140.
Lizeng, Q., Nilsson, C., Sourial, S., Andersson, S., Larsen, O., Aaby, P., Ehnlund, M. , Bjorling, E., 2004. Potent neutralizing serum immunoglobulin A (IgA) in human immunodeficiency virus type 2-exposed IgG-seronegative individuals. J Virol 78, 7016-22.
Logan, S., Tarpley, H.L. , Latimer, K.S. 2004. Caprine Arthritis-Encephalitis Virus.
Magalhaes, P.O., Lopes, A.M., Mazzola, P.G., Rangel-Yagui, C., Penna, T.C. , Pessoa, A., Jr., 2007. Methods of endotoxin removal from biological preparations: a review. J Pharm Pharm Sci 10, 388-404.
Marcello, A., Cinelli, R.A., Ferrari, A., Signorelli, A., Tyagi, M., Pellegrini, V., Beltram, F. , Giacca, M., 2001. Visualization of in vivo direct interaction between HIV-1 TAT and human cyclin T1 in specific subcellular compartments by fluorescence resonance energy transfer. J Biol Chem 276, 39220-5.
Marchio, S., Alfano, M., Primo, L., Gramaglia, D., Butini, L., Gennero, L., De Vivo, E., Arap, W., Giacca, M., Pasqualini, R. , Bussolino, F., 2005. Cell surface-associated Tat modulates HIV-1 infection and spreading through a specific interaction with gp120 viral envelope protein. Blood 105, 2802-11.
Matteucci, D., Pistello, M., Mazzetti, P., Giannecchini, S., Isola, P., Merico, A., Zaccaro, L., Rizzuti, A. , Bendinelli, M., 1999. AIDS vaccination studies using feline immunodeficiency virus as a model: immunisation with inactivated whole virus suppresses viraemia levels following intravaginal challenge with infected cells but not following intravenous challenge with cell-free virus. Vaccine 18, 119-30.
Matteucci, D., Poli, A., Mazzetti, P., Sozzi, S., Bonci, F., Isola, P., Zaccaro, L., Giannecchini, S., Calandrella, M., Pistello, M., Specter, S. , Bendinelli, M., 2000. Immunogenicity of an anti-clade B feline immunodeficiency fixed-cell virus vaccine in field cats. J Virol 74, 10911-9.
Mazanec, M.B., Kaetzel, C.S., Lamm, M.E., Fletcher, D. , Nedrud, J.G., 1992. Intracellular neutralization of virus by immunoglobulin A antibodies. Proc Natl Acad Sci U S A 89, 6901-5.

Mazanec, M.B., Nedrud, J.G., Kaetzel, C.S. , Lamm, M.E., 1993. A three-tiered view of the role of IgA in mucosal defense. Immunol Today 14, 430-5.
Mazzetti, P., Giannecchini, S., Del Mauro, D., Matteucci, D., Portincasa, P., Merico, A., Chezzi, C. , Bendinelli, M., 1999. AIDS vaccination studies using an ex vivo feline immunodeficiency virus model: detailed analysis of the humoral immune response to a protective vaccine. J Virol 73, 1-10.
Mboloi, M.M., Bekker, C.P., Kruitwagen, C., Greiner, M. , Jongejan, F., 1999. Validation of the indirect MAP1-B enzyme-linked immunosorbent assay for diagnosis of experimental Cowdria ruminantium infection in small ruminants. Clin Diagn Lab Immunol 6, 66-72.
McElrath, M.J., Corey, L., Montefiori, D., Wolff, M., Schwartz, D., Keefer, M., Belshe, R., Graham, B.S., Matthews, T., Wright, P., Gorse, G., Dolin, R., Berman, P., Francis, D., Duliege, A.M., Bolognesi, D., Stablein, D., Ketter, N. , Fast, P., 2000. A phase II study of two HIV type 1 envelope vaccines, comparing their immunogenicity in populations at risk for acquiring HIV type 1 infection. AIDS Vaccine Evaluation Group. AIDS Res Hum Retroviruses 16, 907-19.
Meas, S., Usui, T., Ohashi, K., Sugimoto, C. , Onuma, M., 2002. Vertical transmission of bovine leukemia virus and bovine immunodeficiency virus in dairy cattle herds. Vet Microbiol 84, 275-82.
Meiss, G., Friedhoff, P., Hahn, M., Gimadutdinow, O. , Pingoud, A., 1995. Sequence preferences in cleavage of dsDNA and ssDNA by the extracellular Serratia marcescens endonuclease. Biochemistry 34, 11979-88.
Miller, R.J., Cairns, J.S., Bridges, S. , Sarver, N., 2000. Human immunodeficiency virus and AIDS: insights from animal lentiviruses. J Virol 74, 7187-95.
Milman, G. , Sharma, O., 1994. Mechanisms of HIV/SIV mucosal transmission. AIDS Res Hum Retroviruses 10, 1305-12.
Mitola, S., Soldi, R., Zanon, I., Barra, L., Gutierrez, M.I., Berkhout, B., Giacca, M. , Bussolino, F., 2000. Identification of specific molecular structures of human immunodeficiency virus type 1 Tat relevant for its biological effects on vascular endothelial cells. J Virol 74, 344-53.
Montelaro, R.C., Cole, K.S. , Hammond, S.A., 1998. Maturation of immune responses to lentivirus infection: implications for AIDS vaccine development. AIDS Res Hum Retroviruses 14 Suppl 3, S255-9.
Moody, C.A., Pharr, G.T., Murphey, J., Hughlett, M.B., Weaver, C.C., Nelson, P.D. , Coats, K.S., 2002. Confirmation of vertical transmission of bovine immunodeficiency virus in naturally infected dairy cattle using the polymerase chain reaction. J Vet Diagn Invest 14, 113-9.
Moreau, E., Belliard, G., Partidos, C.D., Pradezinsky, F., Le Buanec, H., Muller, S. , Desgranges, C., 2004. Important B-cell epitopes for neutralization of human immunodeficiency virus type 1 Tat in serum samples of humans and different animal species immunized with Tat protein or peptides. J Gen Virol 85, 2893-901.
Nagaraja, T.G., Bartley, E.E., Anthony, H.D., Leipold, H.W. , Fina, L.R., 1979. Endotoxin shock in calves from intravenous injection of rumen bacterial endotoxin. J Anim Sci 49, 567-82.
Naik, S., Renukaradhya, G.J., Rajasekhar, M. , Shaila, M.S., 1997. Immunogenic and protective properties of haemagglutinin protein (H) of rinderpest virus expressed by a recombinant baculovirus. Vaccine 15, 603-7.

Narayan, O., Wolinsky, J.S., Clements, J.E., Strandberg, J.D., Griffin, D.E. , Cork, L.C., 1982. Slow Virus Replication: the Role of Macrophages in the Persistence and Expression of Visna Viruses of Sheep and Goats. J Gen Virol 59, 345-356.

Nenci, C., Zahno, M.L., Vogt, H.R., Obexer-Ruff, G., Doherr, M.G., Zanoni, R., Peterhans, E. , Bertoni, G., 2007. Vaccination with a T-cell-priming Gag peptide of caprine arthritis encephalitis virus enhances virus replication transiently in vivo. J Gen Virol 88, 1589-93.

Noonan, D.M., Gringeri, A., Meazza, R., Rosso, O., Mazza, S., Muca-Perja, M., Le Buanec, H., Accolla, R.S., Albini, A. , Ferrini, S., 2003. Identification of immunodominant epitopes in inactivated Tat-vaccinated healthy and HIV-1-infected volunteers. J Acquir Immune Defic Syndr 33, 47-55.

Oaks, J.L., McGuire, T.C., Ulibarri, C. , Crawford, T.B., 1998. Equine Infectious Anemia Virus is Found in Tissue Macrophages During Subclinical Infection. J Virol 72, 7263-9.

Oaks, J.L., Ulibarri, C. , Crawford, T.B., 1999. Endothelial Cell Infection in Vivo by Equine Infectious Anaemia Virus. Journal of General Virology 80, 2393-2397.

Olmsted, R.A., Langley, R., Roelke, M.E., Goeken, R.M., Adger-Johnson, D., Goff, J.P., Albert, J.P., Packer, C., Laurenson, M.K., Caro, T.M. , et al., 1992. Worldwide prevalence of lentivirus infection in wild feline species: epidemiologic and phylogenetic aspects. J Virol 66, 6008-18.

Orr, K.A., O'Reilly, K.L. , Scholl, D.T., 2003. Estimation of sensitivity and specificity of two diagnostics tests for bovine immunodeficiency virus using Bayesian techniques. Prev Vet Med 61, 79-89.

Osterhaus, A.D., van Baalen, C.A., Gruters, R.A., Schutten, M., Siebelink, C.H., Hulskotte, E.G., Tijhaar, E.J., Randall, R.E., van Amerongen, G., Fleuchaus, A., Erfle, V. , Sutter, G., 1999. Vaccination with Rev and Tat against AIDS. Vaccine 17, 2713-4.

Pancino, G., Camoin, L. , Sonigo, P., 1995. Structural analysis of the principal immunodominant domain of the feline immunodeficiency virus transmembrane glycoprotein. J Virol 69, 2110-8.

Pantaleo, G. , Walker, B.D., 2000. Retroviral Immunology: Immune Response and Restoration. Humana Press, New Jersey.

Pauza, C.D., Trivedi, P., Wallace, M., Ruckwardt, T.J., Le Buanec, H., Lu, W., Bizzini, B., Burny, A., Zagury, D. , Gallo, R.C., 2000. Vaccination with tat toxoid attenuates disease in simian/HIV-challenged macaques. Proc Natl Acad Sci U S A 97, 3515-9.

Pedersen, N.C., Ho, E.W., Brown, M.L. , Yamamoto, J.K., 1987. Isolation of a T-lymphotropic Virus from Domestic Cats with an Immunodeficiency-Like Syndrome. Science 235, 790-793.

Pifat, D.Y., Ennis, W.H., Ward, J.M., Oberste, M.S. , Gonda, M.A., 1992. Persistent Infection of Rabbits with Bovine Immunodeficiency-Like Virus. Journal of Virology 66, 4518-4524.

Poole, S., Mistry, Y., Ball, C., Gaines Das, R.E., Opie, L.P., Tucker, G. , Patel, M., 2003. A rapid 'one-plate' in vitro test for pyrogens. J Immunol Methods 274, 209-20.

Prabowo, H., 1995. Jembrana Disease in Lampung. Jembrana Disease and the Bovine Lentivirus, 96-99.

Pringle, C.R., 1999. Virus taxonomy at the XIth International Congress of Virology, Sydney, Australia, 1999. Arch Virol 144, 2065-70.

Purcell, R.H., Nguyen, H., Shapiro, M., Engle, R.E., Govindarajan, S., Blackwelder, W.C., Wong, D.C., Prieels, J.P. , Emerson, S.U., 2003. Pre-clinical immunogenicity and efficacy trial of a recombinant hepatitis E vaccine. Vaccine 21, 2607-15.
Putra, A.A. , Sulistyana, K., 1995. Epidemiological Observations of Jembrana Disease in Bali. Jembrana Disease and the Bovine Lentivirus, 90-95.
Ramachandran, S., 1995. Early Observations and Research on Jembrana Disease in Bali and on other Islands. Jembrana Disease and the Bovine Lentivirus, 15-25.
Rice, A.P. , Carlotti, F., 1990. Structural analysis of wild-type and mutant human immunodeficiency virus type 1 Tat proteins. J Virol 64, 6018-26.
Roitt, I.R. , Delves, P.D., 2002. Roitts Essential Immunology. Blackwell, London.
Rosati, S., Kwang, J., Tolari, F. , Keen, J., 1994. A comparison of whole virus and recombinant transmembrane ELISA and immunodiffusion for detection of ovine lentivirus antibodies in Italian sheep flocks. Vet Res Commun 18, 73-80.
Rosati, S., Mannelli, A., Merlo, T. , Ponti, N., 1999. Characterization of the immunodominant cross-reacting epitope of visna maedi virus and caprine arthritis-encephalitis virus capsid antigen. Virus Res 61, 177-83.
Rosati, S., Profiti, M., Lorenzetti, R., Bandecchi, P., Mannelli, A., Ortoffi, M., Tolari, F. , Ciabatti, I.M., 2004. Development of recombinant capsid antigen/transmembrane epitope fusion proteins for serological diagnosis of animal lentivirus infections. J Virol Methods 121, 73-8.
Rosen, F.S. , Mackay, I., 2001. The immunology series comes to an end. N Engl J Med 345, 1343-4.
Rothel, J.S., Wood, P.R., Seow, H.F. , Lightowlers, M.W., 1997. Urea/DTT solubilization of a recombinant Taenia ovis antigen, 45W, expressed as a GST fusion protein results in enhanced protective immune response to the 45W moiety. Vaccine 15, 469-72.
Rusche, J.R., Lynn, D.L., Robert-Guroff, M., Langlois, A.J., Lyerly, H.K., Carson, H., Krohn, K., Ranki, A., Gallo, R.C. , Bolognesi, D.P., 1987. Humoral immune response to the entire human immunodeficiency virus envelope glycoprotein made in insect cells. Proc Natl Acad Sci U S A 84, 6924-8.
Rusnati, M., Taraboletti, G., Urbinati, C., Tulipano, G., Giuliani, R., Molinari-Tosatti, M.P., Sennino, B., Giacca, M., Tyagi, M., Albini, A., Noonan, D., Giavazzi, R. , Presta, M., 2000. Thrombospondin-1/HIV-1 tat protein interaction: modulation of the biological activity of extracellular Tat. Faseb J 14, 1917-30.
Saman, E., Van Eynde, G., Lujan, L., Extramiana, B., Harkiss, G., Tolari, F., Gonzalez, L., Amorena, B., Watt, N. , Badiola, J., 1999. A new sensitive serological assay for detection of lentivirus infections in small ruminants. Clin Diagn Lab Immunol 6, 734-40.
Schwarz, E., Lilie, H. , Rudolph, R., 1996. The effect of molecular chaperones on in vivo and in vitro folding processes. Biol Chem 377, 411-6.
Scobie, L., Venables, C., Hughes, K., Dawson, M. , Jarrett, O., 1999. The Antibody Response of Cattle Infected with Bovine Immunodeficiency Virus to Peptides of the Viral Transmembrane Protein. J Gen Virol 80, 237-243.
Scobie, L., Venables, C., Sayers, A.R., Weightman, S. , Jarrett, O., 2001. Prevalence of bovine immunodeficiency virus infection in cattle in Great Britain. Vet Rec 149, 459-60.
Sellon, D.C., Walker, K.M., Russell, K.E., Perry, S.T., Covington, P. , Fuller, F.J., 1996. Equine infectious anemia virus replication is upregulated during

differentiation of blood monocytes from acutely infected horses. J Virol 70, 590-4.
Setiyaningsih, S., Desport, M., Stewart, M.E., Hartaningsih, N. , Wilcox, G.E., 2008. Sequence analysis of mRNA transcripts encoding Jembrana disease virus Tat-1 in vivo. Virus Res 132, 220-5.
Shokri, F. , Jafarzadeh, A., 2001. High seroprotection rate induced by low doses of a recombinant hepatitis B vaccine in healthy Iranian neonates. Vaccine 19, 4544-8.
Soeharsono, S., 1995. Current Information on Jembrana Disease Distribution in Indonesia. Jembrana Disease and the Bovine Lentivirus, 72-75.
Soeharsono, S., Budiantono, A., Sulistyana, K., Tenaya, M., Hartaningsih, N., Dharma, D.M.N., Soesanto, M. , Wilcox, G.E., 1995a. Clinical changes in Bali cattle and other ruminants following infection with Jembrana Disease Virus. Jembrana Disease and the Bovine Lentivirus, 191.
Soeharsono, S., Hartaningsih, N., Soetrisno, M., Kertayadnya, G. , Wilcox, G.E., 1990. Studies of experimental Jembrana disease in Bali cattle. I. Transmission and persistence of the infectious agent in ruminants and pigs, and resistance of recovered cattle to re-infection. J Comp Pathol 103, 49-59.
Soeharsono, S. , Temadja, T.I.G.N., 1995. The Occurence and History of Jembrana Disease in Indonesia. Jembrana Disease and the Bovine Lentivirus, 2-9.
Soeharsono, S., Wilcox, G.E., Dharma, D.M., Hartaningsih, N., Kertayadnya, G. , Budiantono, A., 1995b. Species Differences in the Reaction of Cattle to Jembrana Disease Virus Infection. Journal of Comparative Pathology 112, 391-402.
Soeharsono, S., Wilcox, G.E., Putra, A.A., Hartaningsih, N., Sulistyana, K. , Tenaya, M., 1995d. The Transmission of Jembrana Disease, a Lentivirus Disease of Bos *javanicus* Cattle. Epidemiol Infect 115, 367-74.
Soesanto, M., Soeharsono, S., Budiantono, A., Sulistyana, K., Tenaya, M. , Wilcox, G.E., 1990. Studies on experimental Jembrana disease in Bali cattle. II. Clinical signs and haematological changes. J Comp Pathol 103, 61-71.
Soutullo, A., Verwimp, V., Riveros, M., Pauli, R. , Tonarelli, G., 2001. Design and validation of an ELISA for equine infectious anemia (EIA) diagnosis using synthetic peptides. Vet Microbiol 79, 111-21.
Stauber, R.H. , Pavlakis, G.N., 1998. Intracellular trafficking and interactions of the HIV-1 Tat protein. Virology 252, 126-36.
Stewart, M., Desport, M., Hartaningsih, N. , Wilcox, G., 2005. TaqMan real-time reverse transcription-PCR and JDVp26 antigen capture enzyme-linked immunosorbent assay to quantify Jembrana disease virus load during the acute phase of in vivo infection. J Clin Microbiol 43, 5574-80.
Stittelaar, K.J., Gruters, R.A., Schutten, M., van Baalen, C.A., van Amerongen, G., Cranage, M., Liljestrom, P., Sutter, G. , Osterhaus, A.D., 2002. Comparison of the efficacy of early versus late viral proteins in vaccination against SIV. Vaccine 20, 2921-7.
Straub, O.C. , Levy, D., 1999. Bovine Immunodeficiency Virus and Analogies with Human Immunodeficiency Virus. Leukemia 13, S106-S109.
Streefland, P.H., 2003. Introduction of a HIV vaccine in developing countries: social and cultural dimensions. Vaccine 21, 1304-9.
Suarez, D.L., Van der Maaten, M.J. , Whetstone, C.A., 1995. Improved Early and Long-Term Detection of Bovine Lentivirus by a Nested Polymerase Chain Reaction Test in Experimentally Infected Calves. Am J Vet Res 56, 579-86.

Suarez, D.L., VanDerMaaten, M.J., Wood, C. , Whetstone, C.A., 1993. Isolation and characterization of new wild-type isolates of bovine lentivirus. J Virol 67, 5051-5.
Taube, R., Fujinaga, K., Wimmer, J., Barboric, M. , Peterlin, B.M., 1999. Tat transactivation: a model for the regulation of eukaryotic transcriptional elongation. Virology 264, 245-53.
Tembok, T. , Erinaldi, 1995. Jembrana Disease in West Sumatra. Jembrana Disease and the Bovine Lentivirus, 100-102.
Theisen, D.M., Pongratz, C., Wiegmann, K., Rivero, F., Krut, O. , Kronke, M., 2006. Targeting of HIV-1 Tat traffic and function by transduction-competent single chain antibodies. Vaccine 24, 3127-36.
Tirado, S.M. , Yoon, K.J., 2003. Antibody-dependent enhancement of virus infection and disease. Viral Immunol 16, 69-86.
Tomasulo, P.A., Levin, J., Murphy, P.A. , Winkelstein, J.A., 1977. Biological activities of tritiated endotoxins: correlation of the Limulus lysate assay with rabbit pyrogen and complement-activation assays for endotoxin. J Lab Clin Med 89, 308-15.
Torsteinsdottir, S., Carlsdottir, H.M., Svansson, V., Matthiasdottir, S., Martin, A.H. , Petursson, G., 2007. Vaccination of sheep with Maedi-visna virus gag gene and protein, beneficial or harmful? Vaccine 25, 6713-20.
Torten, M., Franchini, M., Barlough, J.E., George, J.W., Mozes, E., Lutz, H. , Pedersen, N.C., 1991. Progressive immune dysfunction in cats experimentally infected with feline immunodeficiency virus. J Virol 65, 2225-30.
Towbin, H., Staehelin, T. , Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76, 4350-4.
Tyagi, M., Rusnati, M., Presta, M. , Giacca, M., 2001. Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans. J Biol Chem 276, 3254-61.
Uhl, E.W., Heaton-Jones, T.G., Pu, R. , Yamamoto, J.K., 2002. FIV vaccine development and its importance to veterinary and human medicine: a review FIV vaccine 2002 update and review. Vet Immunol Immunopathol 90, 113-32.
van Baalen, C.A., Pontesilli, O., Huisman, R.C., Geretti, A.M., Klein, M.R., de Wolf, F., Miedema, F., Gruters, R.A. , Osterhaus, A.D., 1997. Human immunodeficiency virus type 1 Rev- and Tat-specific cytotoxic T lymphocyte frequencies inversely correlate with rapid progression to AIDS. J Gen Virol 78 (Pt 8), 1913-8.
Van Der Maaten, M.J., Boothe, A.D. , Seger, C.L., 1972. Isolation of a Virus from Cattle with Persistent Lymphocytosis. J. Natl. Cancer Inst. 49, 1649-1657.
Vellutini, C., Horschowski, N., Philippon, V., Gambarelli, D., Nave, K.A. , Filippi, P., 1995. Development of lymphoid hyperplasia in transgenic mice expressing the HIV tat gene. AIDS Res Hum Retroviruses 11, 21-9.
Vellutini, C., Philippon, V., Gambarelli, D., Horschowski, N., Nave, K.A., Navarro, J.M., Auphan, M., Courcoul, M.A. , Filippi, P., 1994. The maedi-visna virus Tat protein induces multiorgan lymphoid hyperplasia in transgenic mice. J Virol 68, 4955-62.
Villet, S., Faure, C., Bouzar, B.A., Morin, T., Verdier, G., Chebloune, Y. , Legras, C., 2003. Lack of trans-activation function for Maedi Visna virus and Caprine arthritis encephalitis virus Tat proteins. Virology 307, 317-27.
Villinger, F., Mayne, A.E., Bostik, P., Mori, K., Jensen, P.E., Ahmed, R. , Ansari, A.A., 2003. Evidence for antibody-mediated enhancement of simian

immunodeficiency virus (SIV) Gag antigen processing and cross presentation in SIV-infected rhesus macaques. J Virol 77, 10-24.
Vives, E., Brodin, P. , Lebleu, B., 1997. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem 272, 16010-7.
Wareing, S., Hartaningsih, N., Wilcox, G.E. , Penhale, W.J., 1999. Evidence for Immunosuppression Associated with Jembrana Disease Virus Infection of Cattle. Vet Microbiol 68, 179-185.
Wedlock, D.N., Denis, M., Skinner, M.A., Koach, J., de Lisle, G.W., Vordermeier, H.M., Hewinson, R.G., van Drunen Littel-van den Hurk, S., Babiuk, L.A., Hecker, R. , Buddle, B.M., 2005. Vaccination of cattle with a CpG oligodeoxynucleotide-formulated mycobacterial protein vaccine and Mycobacterium bovis BCG induces levels of protection against bovine tuberculosis superior to those induced by vaccination with BCG alone. Infect Immun 73, 3540-6.
Whetstone, C., Suarez, D.L., Miller, J.M., Pesch, B.A and Harp, J.A, 1995. Immunological Responses in Cattle(*Bos taurus*) After Inoculation with BIV. Jembrana Disease and the Bovine Lentivirus, 138-143.
Whetstone, C.A., Suarez, D.L., Miller, J.M., Pesch, B.A. , Harp, J.A., 1997. Bovine Lentivirus Induces Early Transient B-Cell Proliferation in Experimentally Inoculated Cattle and appears to be Pantropic. J Virol 71, 640-644.
Whetstone, C.A., VanDerMaaten, M.J. , Black, J.W., 1990. Humoral immune response to the bovine immunodeficiency-like virus in experimentally and naturally infected cattle. J Virol 64, 3557-61.
Wilcox, G.E., 1997. Jembrana disease. Aust Vet J 75, 492-3.
Wilcox, G.E., Chadwick, B.D. , Kertayadnya, G., 1995. Recent Advances in the Understanding of Jembrana Disease. Vet Microbiol 46, 249-255.
Wiryosuhanto, S., 1995. Bali Cattle-Their Economic Importance in Indonesia. Jembrana Disease and the Bovine Lentivirus, 34-41.
Wu, Z., Liu, S., Zhang, S., Tong, H., Gao, Z., Liu, Y., Lin, D., Liu, Z., Wu, G., Yi, H., Song, G. , Xu, Y., 2004. Persistence of the protective immunity to Schistosoma japonicum in Chinese yellow cattle induced by recombinant 26kDa glutathione-S-transferase (reSjc26GST). Vet Parasitol 123, 167-77.
Xie, B., Calabro, V., Wainberg, M.A. , Frankel, A.D., 2004. Selection of TAR RNA-binding chameleon peptides by using a retroviral replication system. J Virol 78, 1456-63.
Xie, B., Wainberg, M.A. , Frankel, A.D., 2003. Replication of human immunodeficiency viruses engineered with heterologous Tat-transactivation response element interactions. J Virol 77, 1984-91.
Xuan, C., Qiao, W., Li, J., Peng, G., Liu, M., Chen, Q., Zhou, J. , Geng, Y., 2008. BTat, a trans-acting regulatory protein, contributes to bovine immunodeficiency virus-induced apoptosis. Cell Microbiol 10, 31-40.
Yamamoto, J.K., Okuda, T., Ackley, C.D., Louie, H., Pembroke, E., Zochlinski, H., Munn, R.J. , Gardner, M.B., 1991. Experimental vaccine protection against feline immunodeficiency virus. AIDS Res Hum Retroviruses 7, 911-22.
Yang, Y., Tikhonov, I., Ruckwardt, T.J., Djavani, M., Zapata, J.C., Pauza, C.D. , Salvato, M.S., 2003. Monocytes treated with human immunodeficiency virus Tat kill uninfected CD4(+) cells by a tumor necrosis factor-related apoptosis-induced ligand-mediated mechanism. J Virol 77, 6700-8.

Yin, J., Li, G., Ren, X. , Herrler, G., 2007. Select what you need: a comparative evaluation of the advantages and limitations of frequently used expression systems for foreign genes. J Biotechnol 127, 335-47.

Zhang, H., Troyer, D.L., Kapil, S., Zheng, L., Kennedy, G., Weiss, M., Xue, W., Wood, S. , Minocha, H.C., 1997a. Detection of Proviral DNA of Bovine Immunodeficiency Virus in Bovine tissues by Polymerase Chain Reaction (PCR) and PCR *in situ* Hybridization. Virology 236, 249-257.

Zhang, S., Wood, C., Xue, W., Krukenberg, S.M., Chen, Q. , Minocha, H.C., 1997b. Immune suppression in calves with Bovine Immunodeficiency Virus. Clin Diagn Lab Immunol 4, 232-235.

Zhang, Y. , Barkalis, E., 1997. Effects of Nucleocapsid Mutations on Human Immunodeficiency Virus Assembly and RNA Encapsidation. J Virol 71, 6765-6776.

Zheng, L., Swanson, M., Liao, J., Wood, C., Kapil, S., Snider, R., Loughin, T.A. , Minocha, H.C., 2000. Cloning of the bovine immunodeficiency virus gag gene and development of a recombinant-protein-based enzyme-linked immunosorbent assay. Clin Diagn Lab Immunol 7, 557-62.